Studies into the structural and functional integration of the chloroplast.

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STUDIES INTO THE STRUCTURAL AND FUNCTIONAL INTEGRATION OF THE CHLOROPLAST

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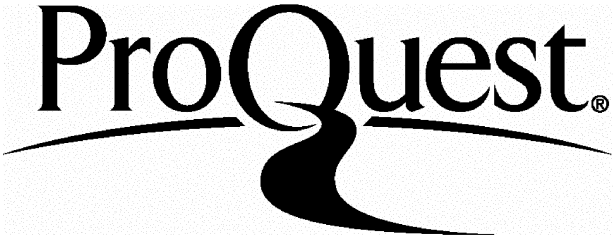
A. Robertson.



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ABBREVIATIONS

ADP	- Adenosine diphosphate
ALA	- Aminolevulinic acid
ATP	- Adenosine triphosphate
C_4 plants	- Plants that produce a 4-carbon dicarboxylic acid as the first product of photosynthetic CO_2 fixation
CMU	- 3-(4-chlorophenyl)-1,1-dimethylurea
DAB	- Diaminobenzidine
DCIP	- Dichlorophenol indolphenol
DCMU	- 3-(3,4, dichlorophenyl)-1,1-dimethylurea
DEAE	- Diethylaminoethyl
DPC	- Diphenyl Carbazide
DNA	- Deoxyribonucleic acid
EDTA	- Ethylenediaminetetra acetic acid
E'o	- Oxidation-reduction potential at pH 7.0
FRS	- Ferredoxin reducing substance
FMN	- Flavine mononucleotide
IRGA	- Infra red gas analyser
$NADP^+$	- Nicotinamide adenine dinucleotide phosphate
NADPH	- Reduced nicotinamide adenine dinucleotide phosphate
OD	- Optical density
Pi	- Inorganic phosphate
PMS	- Phenazine methosulphate
PS I	- Photosystem I
PS II	- Photosystem II

RuDP Carboxylase	- Ribulose 1,5, diphosphate carboxylase (Carboxydismutase)
Sandoz 6706	- 4-chloro-5-(dimethylamino)-2-(α,α,α-trifluoro-m-tolyl)-3(2H)-pyridazione
SDS	- Sodium dodecyl sulphate
SiMo	- Silicomolybdate
TCA	- Trichloroacetic acid
TEMED	- NNN'N' Tetramethylethylene diamine
TLC	- Thin layer chromatography
TNBT	- Tetranitro Blue Tetrazolum

SUMMARY

Etiolated pea explants were greened in the presence of the photosynthetic inhibitor CMU. Chlorophyll synthesis, except for the initial photoconversion of protochlorophyll(ide) to chlorophyll, was totally inhibited. In addition, the development of chloroplast fine structure subsequent to the prolamellar body dispersal was also inhibited, and the plastids remained functionally inactive. The addition of exogenous sucrose reversed the inhibition of both chlorophyll synthesis and fine structural development. Morphologically normal chloroplasts were formed which contained chlorophyll levels above those of the water control. Measurement of CO_2 uptake demonstrated that leaves treated in this manner remained photosynthetically incompetent. This experimental procedure was utilised in order to compare the relationship between functional and structural development of the chloroplast in normal and functionally incompetent leaves, and to assess the exact contribution of photosynthesis towards this development.

The results showed that the pigment composition of the CMU/sucrose treated leaves developed normally, but PS II activity, as measured by ferricyanide reduction remained inactive. Manganese incorporation into these chloroplast membranes suggested that the oxygen evolving centres of PS II were developing in the absence of electron flow, and this was confirmed by detection of PS II activity through silicomolybdate reduction. It is concluded that, provided a

substrate such as sucrose is present, the components associated with a functional PS II will be synthesised in the absence of photosynthetic electron flow.

Similar conclusions were drawn in relation to the development of PS I, as measured by ascorbate photooxidation. However, measurement of PS I activity as $NADP^+$ reduction demonstrated a 50% decrease in rates compared with those of the control. The synthesis of ferredoxin-$NADP^+$reductase is suggested to require an intact photosynthetic electron transport system, which cannot be replaced by an exogenous substrate.

In vitro PMS-mediated cyclic photophosphorylation was detected in chloroplasts isolated from CMU/sucrose treated explants. In addition, evidence from light-stimulated chlorophyll synthesis in these explants supported the existence of in vivo cyclic phosphorylation. The role of this process in chloroplast development is discussed.

A correlation of the onset and development of various portions of chloroplast electron transport with changes in fine structure, confirmed the theory that the organisation of the photosynthetic membrane is a predetermined multistep assembly process. The instigation of PS II was preceded by PS I which appeared to be formed from etioplast components. The synthesis of components associated with PS II was shown to correspond with grana formation, and the relationship between these two events is discussed.

Two examples of PS I activation when PS II was impaired, have been outlined and discussed in relation to the concept of excitation energy spillover.

INTRODUCTION

INTRODUCTION

Photosynthesis in plants may be defined as the light driven evolution of oxygen from water and the storage of the resultant reducing power in organic carbon compounds. Thus solar energy, stored in the form of carbohydrates, is the basis for all autotrophic and heterotrophic organisms and a constant recycling of oxygen is vital for aerobic life.

The photosynthetic process takes place in chlorophyll containing organelles, known as chloroplasts, which are situated within the cytoplasm of higher plant and animal cells. The overall morphology of chloroplasts may vary considerably depending on the organism studied, but the typical higher plant chloroplast is ellipsoidal. The most prominent feature of the chloroplast is its internal double membrane system which is now known to contain all the components associated with the light reaction of photosynthesis. Elucidation of the molecular architecture of this membrane, and a knowledge of its components, could greatly enhance our present understanding of the photosynthetic process. The advent of the electron microscope and associated techniques has produced a means by which the chloroplast membrane can be studied and consequently the pure mechanistic approach to photosynthetic research has given way, in part, to correlative structural and functional studies. The phenomenon of etiolation has added greatly to this method of approach since the development of the chloroplast from photochemically inactive etioplasts

can be controlled and studied in relation to structural changes.

In subsequent sections of this introduction an attempt has been made to review past and present functional and structural studies of the chloroplast and where it has been made possible, to correlate the two.

1. PLASTID FINE STRUCTURE

1.1 The chloroplast

The typical higher plant chloroplast is ellipsoidal in shape with a major diameter between 3 - 10 μm. Electron micrographs of sectioned chloroplasts show them to be bounded by a double membrane, each 20 Å thick and enclosing a space 10 Å wide. Contained within the chloroplast 'envelope' is a proteinaceous matrix called the stroma, which supports a number of particulate structures including ribosomes and strands of DNA. The stroma centre, first discovered by Gunning (1965) appears as a mass of tightly packed, but regularly orientated fibrils. Each fibril has a subunit structure closely related to that of RuDP Carboxylase (Gunning et al, 1967). Present also within the stroma are osmiophillic globules, which on purification appear to be composed of chloroplast quinones such as plastoquinone, Vitamin K_1 and other lipids.

The unique feature of the chloroplast is its internal lamellar system which appears to be composed of numerous flattened sacs or

cisternae called thylakoids (Menke, 1962). Although the development of terminology used to describe the lamellar system has been diverse, the system of nomenclature used here and subsequently is based on that of Menke (1962) and Weier and coworkers (1963; 1965). The thylakoids occur in regular closely-packed stacks called grana, and have been likened to piles of coins. The end portion of each thylakoid which is in contact with the stroma, is called the margin. A partition is the electron dense region where two thylakoids are tightly appressed and the loculus is the internal space enclosed by one thylakoid. Many of the grana lamellae extend out from either or both sides of the grana stack into the stroma to connect with other grana. These interconnecting lamellae are called stroma lamellae.

Due to the large focal depth of the electron microscope, the three dimensional picture of the chloroplast was slow to emerge. Initial three dimensional visualisations, obtained by indirect means were proposed by Heslop-Harrison (1962) and Weier et al., (1963)(figures 1B and 1C). This led Heslop-Harrison (1963) to suggest that the "entire lamellar system of the chloroplast, including all the grana, constitutes a single, enormously complex, membrane bounded entity, separate and distinct from the stroma." This idea was persued by Wehrmeyer (1964) who found that a single stroma lamellae could exist in a spiral arrangement around a granum, being connected with the individual granum thylakoids (figure 1A). Further elaboration by Paolillo and coworkers (1966; 1967a and b; 1969) has resulted in the latest schematic representation (Paolillo, 1970) based on seven species of flowering plant (figure 1D). He suggested that the spiral arrangement of the stroma lamellae around the granum was as an ascending right handed helix, and that each compartment of the

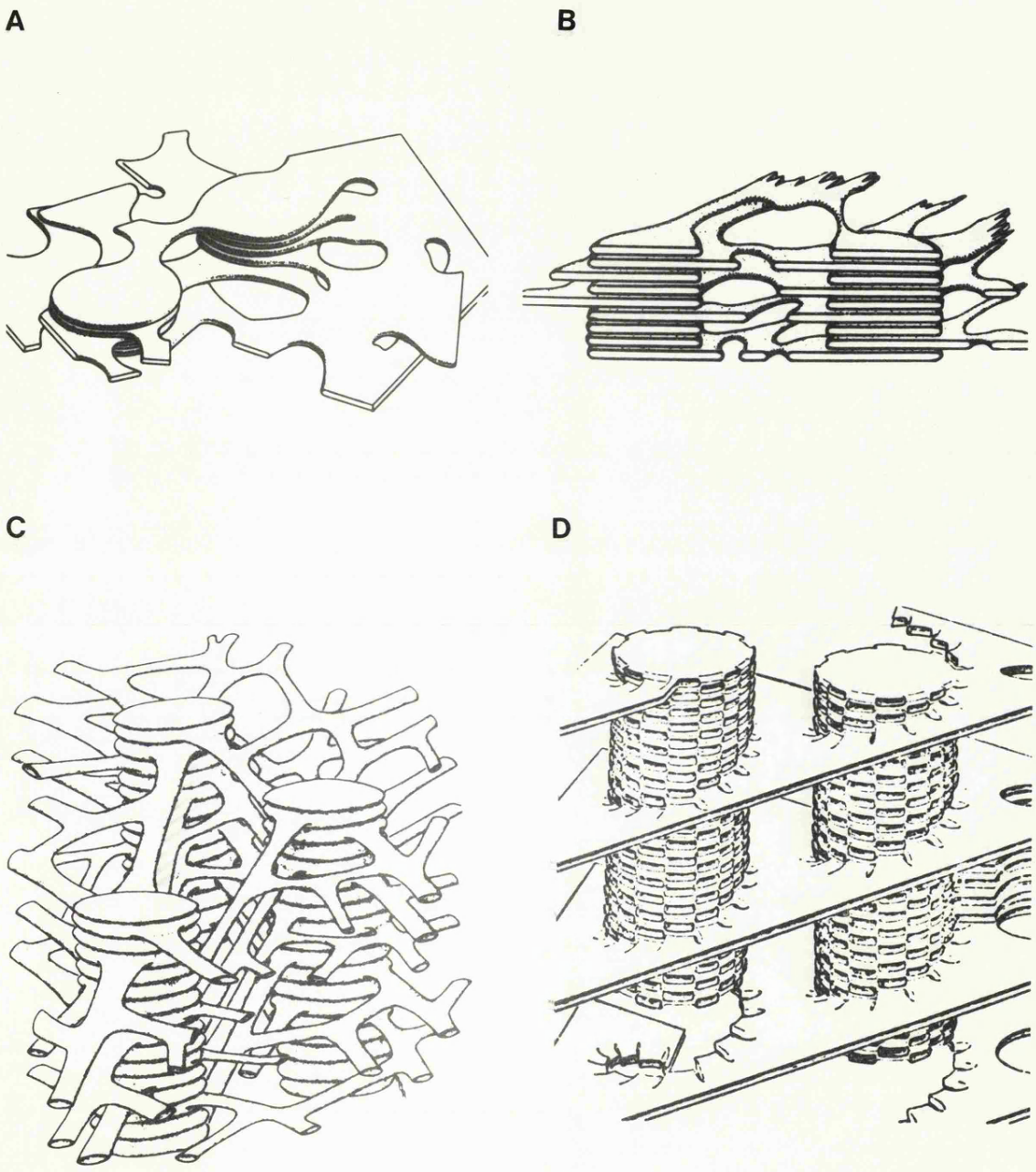


Figure 1. Diagrammatic representation of the possible three-dimensional structure of the internal membrane from a mature chloroplast. (A) from Wehrmeyer, 1964. (B) Heslop-Harrison, 1962. (C) Weier et al., 1963. (D) Paolillo,1970.

granum could contact eight different stroma lamellae, which in turn interconnected with all other grana. In this manner the total lamellar was composed of one or a few folded thylakoids. This arrangement of the internal lamellae system does suggest as first noted by Weier et al, (1966) that the loculus could function as a channel for diffusion of various photosynthetic substrates.

1.2 Etioplast

Etiolation, caused by growing plants in darkness, results in a variety of typical morphological characteristics such as increased stem elongation and the production of yellow leaves. The normal processes which in illuminated plants lead to the development of green, photosynthetically competent chloroplasts are diverted to produce characteristic etioplasts. In place of the normal grana sturcture of thylakoids there is now an internal membrane system arranged in a semi-crystalline lattice (Heitz, 1954) which is known as the prolamellar body.

In three dimensions the prolamellar body is composed of repeating units of two basic types. The first is a hexahedral (figure 2A) in which the membrane tubes branch at right angles, so as to point outwards in six directions. The prolamellar lattice formed from this unit is cubic (Gunning ,1965) and is therefore the most simple arrangememt (figure 2B). The second and most common is the tetrahedral unit as shown in figure 2C (Wehrmeyer, 1967; Weier and Brown, 1970). The latter may interconnect to form many types of

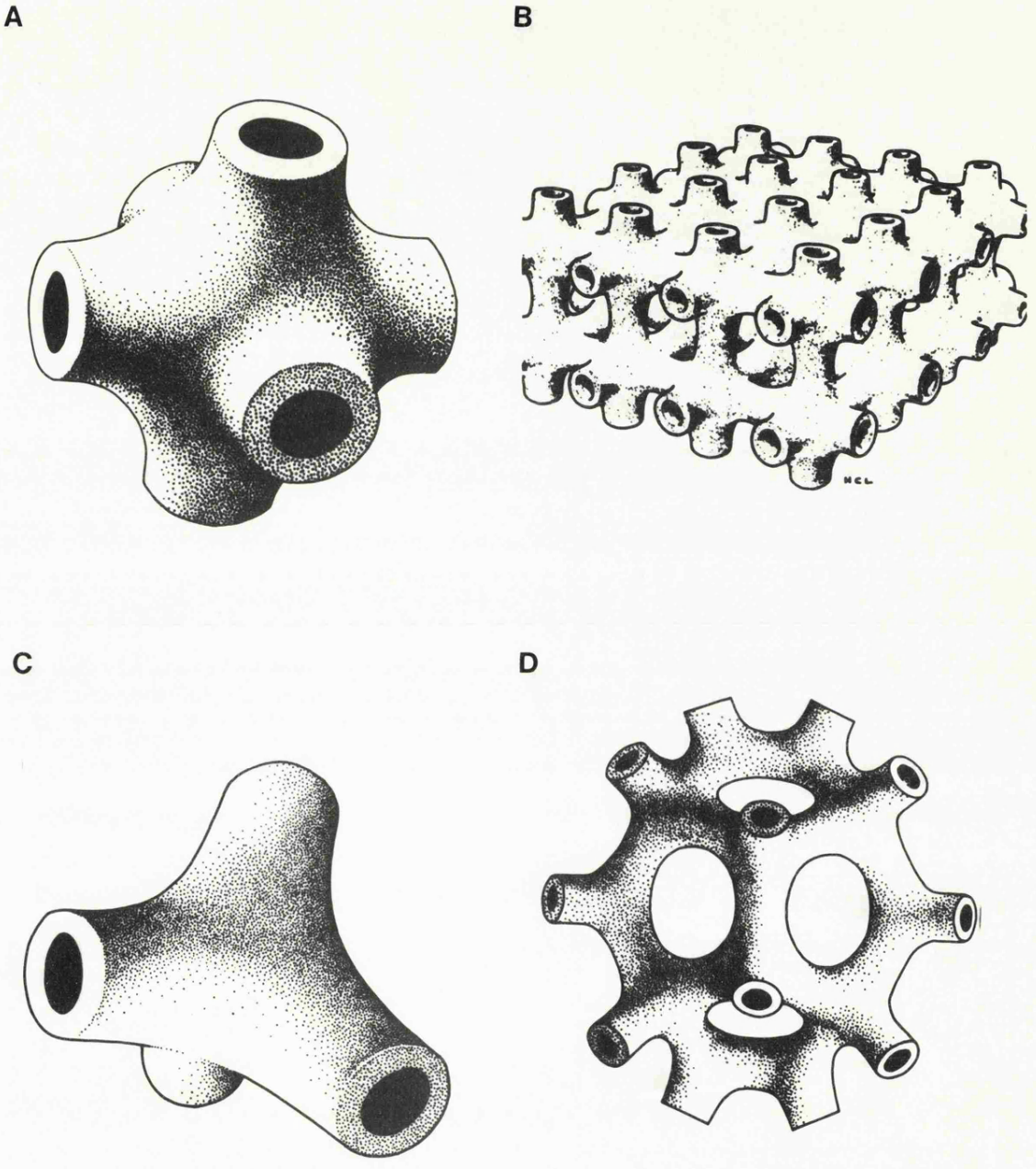


Figure 2. Proposed structures for the arrangement of membranes within the prolamellar body. Hexahedral units (A) form the simple prolamellar lattice (B). The more common tetrahedral unit (C) interconnects to form the basic pentagonal dodecahedron (D). (Gunning and Steer, 1975).

arrangement most of which are based on the pentagonal dodecahedron (figure 2D). The different types of arrangement are not restricted to any particular species of plant etioplast but may be found in any one prolamellar body.

The high volume to surface area of the prolamellar body suggest its function to be one of membrane storage. However, other configurations providing more efficient membrane storage would indicate that this is not its sole function. The complexity of the prolamellar body allows also for stability and for the greatest surface area of membrane to be in contact with the etioplast stroma. The necessity for contact between membrane and stroma implies that membrane components are possibly synthesised within the stroma and can be inserted at any point. It is significant that ribosomes within the stroma component of the prolamellar body are commonly seen (Gunning and Jagoe, 1967). The incorporation of protochlorophyll into its protein holochrome is thought to occur at this stage and is the initial event in a multistep assembly process of chloroplast lamellae (see reviews by Siekevitz et al, 1967; Kirk, 1970).

Illumination of etiolated plants (see section 1.3) results in the dispersal of the prolamellar body, and its gradual transformation into typical chloroplast thylakoids.

1.3 Ultrastructural changes during the development of etioplast into chloroplast

When the etiolated leaf is exposed to light the etioplasts undergo metamorphosis into chloroplasts. It is during this period of transition that the synthesis of chlorophyll and the development of the photochemistry occurs.

The initial ultrastructural change appears to be a loss in the crystallinity of the prolamellar body, although continuity of the membrane surface is retained (Eriksson et al., 1961; Virgin et al., 1963). Low intensity illumination or high intensity flash illumination (Eriksson et al., 1961) particularly in the red and blue regions of the spectrum (Virgin et al., 1963) will produce this change. This data, in addition to action spectrum data, suggest that protochlorophyllide may be the primary photoreceptor.

The rapid, light induced, reduction of protochlorophyllide (see section 2.2) appears to coincide with this initial ultrastructural change. The two processes may not, however, go hand in hand. Virgin et al.(1963) showed that 61% of the protochlorophyllide could be photoreduced without ultrastructural change. More recent studies have shown that prolamellar bodies may be reformed in high (Treffry, 1973) and low (Weier et al., 1970; Henningsen and Boynton, 1970) light intensities without concomitant protochlorophyllide reversal.

Further illumination causes the tubular connections in the prolamellar body to 'pinch off' and the resultant lamellae disperse in the stroma as parallel, two-dimensional, double membrane sheets (Gunning

and Jagoe, 1965). The area of thylakoid produced during this process is not thought to exceed the area of membrane in the prolamellar body, and is therefore a rearrangement of existing membrane, rather than a new synthesis (Bradbeer, 1973; Gunning and Jagoe, 1965; Henningsen and Boynton, 1969).

The primary lamellae are perforated by numerous pores, and closely resemble the perforated thylakoids that are thought to be precursor material for the prolamellar body. The pores are, therefore, the relics of the lattice spacings in the prolamellar body, whereas in the conversion of proplastids to etioplasts, they may well be the progenitors of the lattice spacings. The pores or perforations eventually disappear with prolonged illumination, giving rise to featureless primary thylakoids. The latter have been correlated with the end of lag phase during which membrane extension occurs (Kirk and Tilney-Bassett, 1967). Prolamellar body dispersal is light dependent, proceeding at intensities as low as 20 lux (Eilam and Klein, 1962). The rate of extrusion is proportional to light intensity and with sufficient intensity, will be complete within several minutes (Virgin et al., 1963). The photoreceptor is at present elusive although it has a very specific absorption maximum at 470 nm (Henningsen, 1967).

The end of the lag phase is reflected ultrastructurally by granal formation. The production of the latter from the primary thylakoid is a complex phenomenon not yet fully understood. However, it is thought that newly synthesized membrane materials form a pouch which protrudes from one face of the primary thylakoid (Heslop-Harrison, 1966; Kirk and Tilney-Bassett, 1967; Wehrmeyer, 1964). Rather than

growing out at an angle, the new thylakoid turns and slides over the surface of the parent thylakoid. The former extends until it becomes a circular disc, connected by a narrow neck, through which the continuity of the parent and daughter intrathylakoid space is maintained.

This process is repeated and a second granum thylakoid slides over the first, in such a way that it forms the beginning of the right hand helix as shown in figure 1A. The process is termed spirocyclic growth and will continue until the maximum number of grana per stack is reached for any species. The factor(s) controlling the amount and area of the grana thylakoids is unknown.

Differentiation of etioplasts to chloroplasts in the leaves of Phaseolus vulgaris during continuous illumination, is complete after 45h (Klein et al., 1964) although this may vary with the age and species of the plant.

2. THE CHLOROPHYLLS

2.1 General characteristics

The energy available for photosynthesis in the plant originates from the sun. Passage through the earth's atmosphere results in the total depletion of wavelengths below 300 nm. The radiation available to higher plants and algae, therefore, lies mainly within the visible and short infra-red wavelengths (300 - 900 nm).

The action spectrum for photosynthesis reveals that the

chlorophylls are the major light harvesting pigments of higher plants and algae, with absorption maxima in the red and blue regions of the spectrum. They are metallocompounds each consisting of four substituted pyrrole rings (see figure 3) arranged cyclicly so that the nitrogen atoms are complexed with magnesium. A long chain aliphatic alcohol (phytol) is esterified to ring IV, the removal of which results in the formation of chlorophyllide. Protochlorophyllide is the oxidation product of the latter, and is the terminal dark synthesised chlorophyll intermediate found in etioplast prolamellar bodies.

Higher plants and algae contain two kinds of chlorophyll. Chlorophyll a is the predominant pigment and is present in all oxygen evolving organisms. Derivative spectrophotometry, low temperature absorption measurements and action spectra suggest that chlorophyll a is present in several different forms with absorption maxima at 660, 670, 680, 685, 690 and 700 - 720 nm. It would appear that all forms are the same molecule but that the orientation within the chloroplast membrane results in the different absorption maxima.

In addition to chlorophyll a, two forms of chlorophyll b are also present in higher plants and algae (Shlyk et al., 1970). Chlorophyll b differs from the former in that it has an aldehyde as opposed to a methyl group at C_3, ring II. Although not conclusively proven it is believed that chlorophyll b is synthesised from chlorophyll a (Shlyk et al., 1972). The arrangement of the various chlorophylls within the photosystems may be seen in sections 4.3.1 and 4.4.1.

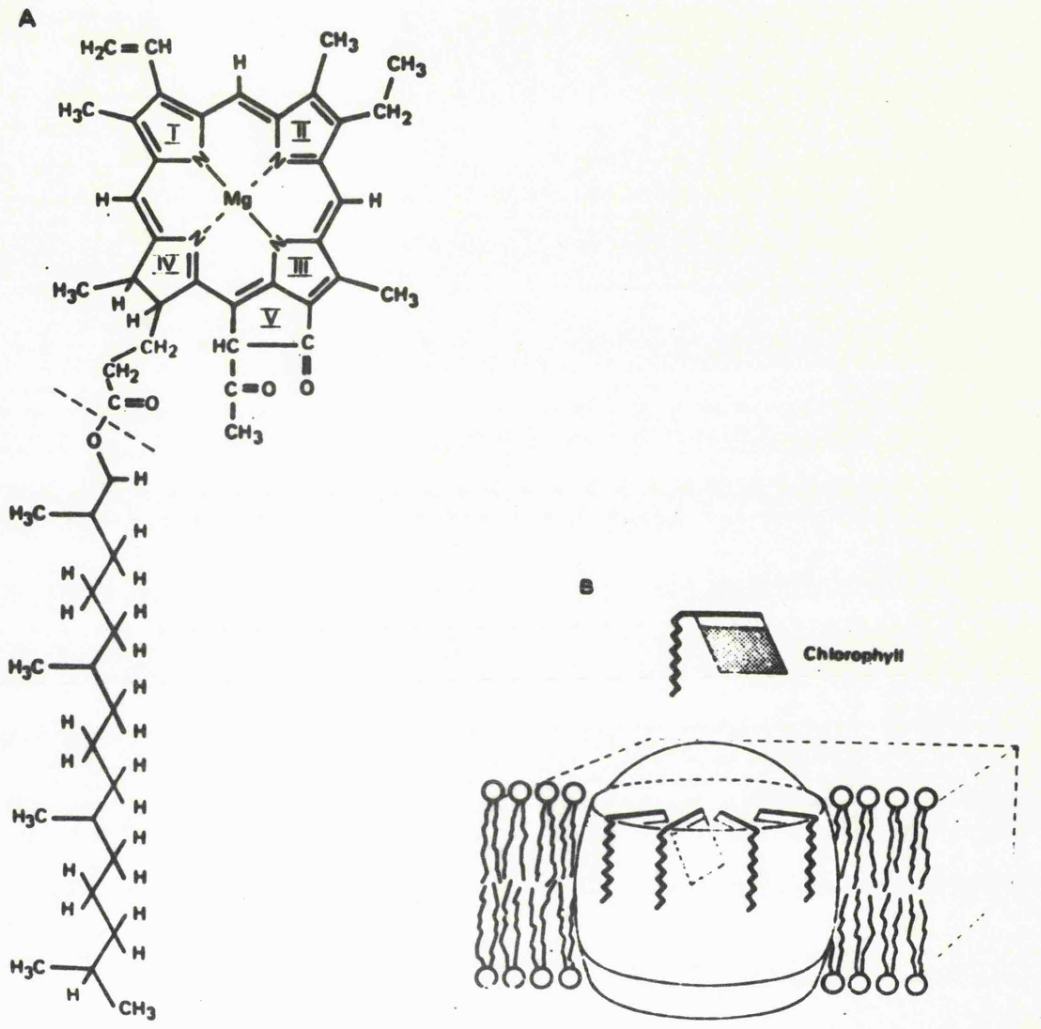


Figure 3. A: The chlorophyll a molecule. B: Diagram showing the possible location of chlorophyll molecules within an intrinsic protein which is spanning the membrane. The hydrophilic regions of the protein are located at the membrane surfaces and the hydrophobic region is embedded within the nonpolar interior of the membrane lipid bilayer. The phytyl chains of the chlorophyll molecules are perpendicular to the membrane surface in close interaction with the outside perimeter of the hydrophobic region of the intrinsic protein. The chlorin rings are buried within the protein so that hydrophilic edge of the chlorin ring is integrated with the hydrophobic interior of the intrinsic complex (From Anderson, 1975).

2.2 Protochlorophyll and its photoconversion

The active form of protochlorophyll exists in association with a protein molecule of molecular weight 600,000 - 700,000 (Smith, 1960; Boardman, 1962), and is referred to as the protochlorophyll holochrome (Smith, 1960). The incorporation of protochlorophyll into the holochrome has been placed before phytylation (Granick, 1967), and the association appears to be maintained throughout subsequent conversions of protochlorophyll to chlorophyll.

Protochlorophyll exists in two forms, phytylated and non-phytylated (Loeffler, 1955). To avoid any confusion the terms protochlorophyllide ester and protochlorophyllide are used to describe the two forms. When there is some doubt as to which form of the pigment the generic term protochlorophyll is used (after Kirk and Tilney-Bassett, 1967).

Spectral and fluorescence studies have shown that protochlorophyllide has a maximum absorbance peak at 650 nm (Shabata, 1957) and a fluorescence peak at 655 nm (Litvin and Krasnovsky, 1957), both of which change upon illumination. Similar studies on isolated protochlorophyllide ester show its absorption and fluorescence maxima to peak at 636 and 633 nm respectively, and these remain stable upon illumination. These data and that of others (Wolfe and Price, 1957; Virgin, 1960), strongly suggest that protochlorophyllide is photoreducable to chlorophyllide whereas protochlorophyllide ester is inactive.

The recent studies of Boardman et al., (1970), have provided evidence that protochlorophyllide ester A636 is, in fact, a mixture of

two species of protochlorophyll with absorption maxima at 628 and 637 nm respectively. Protochlorophyll A628 is present in minor amounts and is photostable. Protochlorophyllide A637 is generally present in approximately equal proportions with protochlorophyllide A650 and together comprise 85 - 90% of the total protochlorophyll present in the plastid. Both are photoconvertible.

When etiolated leaves are illuminated, protochlorophyllide is rapidly converted to chlorophyllide (Koski, 1950). The process is accompanied by a concomitant absorption change from 650 to 684 nm (Shabata, 1957). During a subsequent dark period an absorption shift, from 684 nm to 673 nm, is observed. More recent spectral and fluorescence studies have shown that there are two other intermediates, chlorophyllide A668 (Litvin and Belyaeva, 1968), and chlorophyllide A676 (Gassman et al., 1968; Sironval et al., 1968a). The probable order and subsequent conversion of these chlorophyll intermediates is shown in figure 4. Two consecutive light reactions yield firstly chlorophyllide A668 which is then converted to chlorophyllide A683 - 684.

The subsequent slow (14 - 30 min) 'Shabata' Shift of chlorophyllide A683 to chlorophyllide A673 is accompanied by simultaneous phytylation of the latter to chlorophyll 673. Phytylation is not, however, thought to be the direct reason for the absorption change (Boardman, 1966; Schopfer and Siegelman, 1968; Akoyunoglou and Michalopoulos, 1971), but rather due to conformational membrane changes (Ballschmiter and Katz, 1968). Large amounts of chlorophyll 673 and 683 are synthesised following the lag phase, and this leads to a red shift at 677 nm (Butler,1966; Cederstrand et al., 1966).

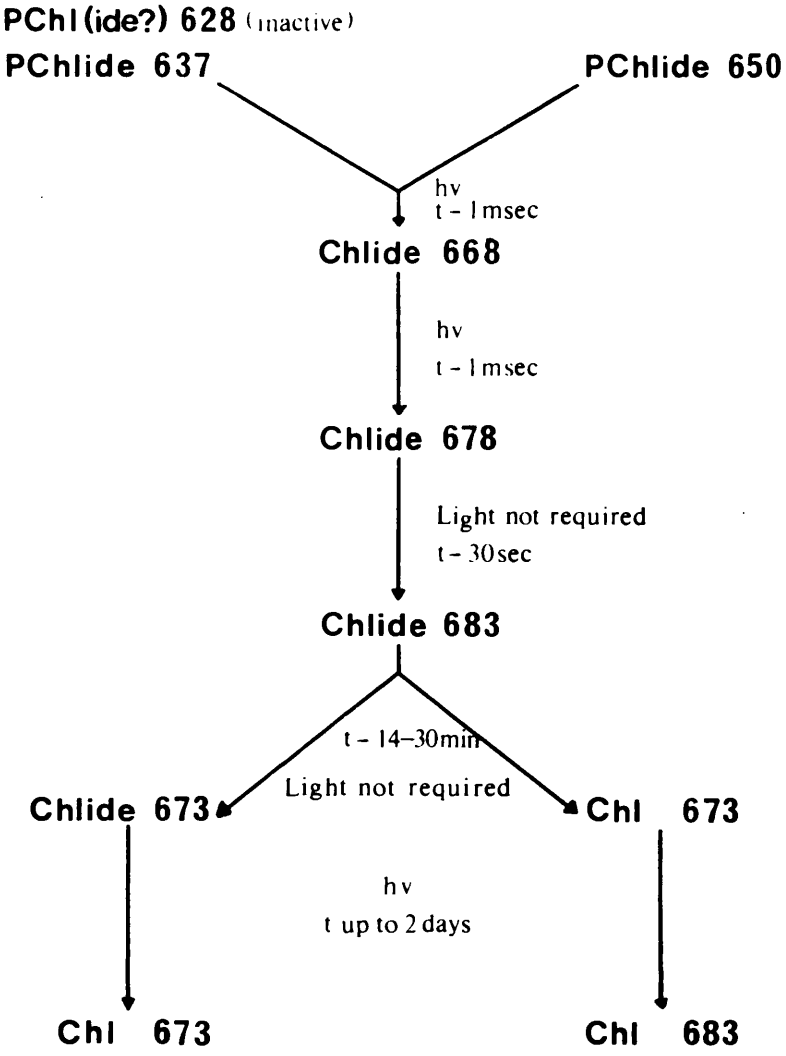


Figure 4. Flow diagram showing the spectroscopic changes in protochlorophyll(ide) and chlorophyll(ide) detectable at different stages of greening. The position (nm) of the in vivo absorption peak is denoted by the number following the name of each pigment.

2.3 Time course of chlorophyll synthesis

The rapid conversion of protochlorophyllide, present in the prolamellar body, to chlorophyllide is followed by a lag period, normally lasting 1 - 2h during which little or no chlorophyll is synthesised (Koski, 1950). The length of the lag phase may be significantly increased in explants, by the removal of endogenous substrates during dark incubation in distilled water (Wolfe and Price, 1957). This may be reversed by the exogenous addition of glucose or sucrose to the incubation medium, thereby substituting for the absent cotyledons. In young leaves the chlorophyll lag phase is not apparent, but increases with age (Sisler and Klein, 1963). Exogenously applied carbohydrates (Palladin, 1923; Sisler and Klein, 1963) and δ-ALA (Sisler and Klein, 1963) have been observed to reduce this lag phase. Hence, it has been concluded that the lag phase is a feature of substrate shortage for chlorophyll synthesis. In some circumstances, however, substrate addition will not restore normal chlorophyll synthesising capacity. Sisler and Klein (1963) observed an increase in chlorophyll synthesising capacity in Phaseolus vulgaris between the fourth and fifth days of growth. Removal of the leaves on the fourth day, and incubation with exogenous substrates, was insufficient to produce chlorophyll synthetic rates associated with the 5 day old leaves. The possible involvement of growth regulators has been proposed to explain these results (Hole, 1973).

A short red light flash, followed by a dark period, prior to illumination with white light will abolish the lag phase (Withrow et

al., 1956), not later than 9h after the initial red flash (Price and Klein, 1961).

Abolition of the lag phase using high humidity growth conditions has recently been observed (Alberte et al., 1972). The availability of water appears, therefore, to be a prerequisite for developing chloroplasts. The lag phase is followed by a period of active chlorophyll synthesis, during which the chloroplast develops in both function and structure. The chlorophyll level eventually reaches a steady state, due probably to the fact that the rate of chlorophyll synthesis is equal to that of chlorophyll destruction or photooxidation.

2.4 The chemical environment of chlorophyll

The Danielli-Davson model proposed for membrane structure (Danielli and Davson, 1935) postulates a lipid monolayer sandwiched between two protein layers. Hanson (1939) argued, however, that there was insufficient space for the chlorophyll molecules to spread out as a monolayer and postulated that the chlorin rings must be tilted with respect to the membrane surface. Dichroism and fluorescence measurements led Kreutz (1968, 1970) to verify these ideas and to suggest also that the phytyl chains of chlorophyll molecules were inserted adjacent to the outside perimeter of the hydrophobic portion of two chlorophyll-protein complexes (see this section) and would therefore be part of a boundary lipid (see figure 3B). The tetrapyrrole moiety was thought to be angled so that the more hydrophilic portion reacted with the hydrophilic membrane surface and the hydrophobic part was

buried in the hydrophobic region of the intrinsic protein. This model favours the existence of chlorophyll on one surface of the membrane for efficiency of light trapping. Briantais et al. (1972; 1973), however, favour the view that the chlorin rings are located on opposite sides of the membrane.

The first evidence that chlorophyll was complexed with protein was obtained by Smith and Pickels (1941), after treatment of chloroplasts with the anionic detergent SDS. The results of this work led them to suggest the existence of one chlorophyll complex containing a chlorophyll a:b ratio of 3:1. Subsequent experiments using electrophoretic separation techniques presented evidence that the chlorophylls were located in more than one molecular environment within the membrane (Chiba, 1960; Sironval et al., 1967; Boardman and Anderson, 1964).

The advent of polyacrylamide gel electrophoresis of anionic detergent extracts, provided substantial evidence for the presence of two major chlorophyll-protein complexes in higher plants (Ogawa et al., 1966; Thornber et al., 1966). Three chlorophyll containing bands can be resolved by this method and will be termed complexes I, II and III according to the increasing order of mobility. Analysis of each of the pigment bands has shown complex III to be the free pigment, derived from the other two pigmented bands (Thornber et al., 1967).

The chlorophyll a:b ratio of chlorophyll-protein complex I, as measured by conventional spectrophotometry, has been calculated to be >8:1 (Shiozawa et al., 1974; Thornber et al., 1967). Other more reliable methods, however, show a complete absence of chlorophyll b.

Chlorophyll a and β-carotene, in a molecular ratio of 20 - 30:1, account therefore, for all the pigment in the complex (Shiozawa et al.,1974). The native protein from complex I has a molecular weight of approximately 110,000 daltons (Eaglesham and Ellis, 1974; Kung and Thornber, 1971), with a chlorophyll:protein ratio of 14 moles chlorophyll/110,000g complex (Thornber, 1969). P700 has also been identified in this complex, in the P700:chlorophyll ratio of 1:40 - 50 (Dietrich and Thornber, 1971; Shiozawa et al., 1974). It is therefore probable that not every complex I unit contains a P700 molecule. Hence there are possibly two different complexes in a homogenous preparation, but at present electrophoretic techniques are not able to resolve them (Dietrich and Thornber, 1971). On the basis of this pigment composition it is thought that the chlorophyll-protein complex I is the heart of PS I, which in addition has been demonstrated to cycle electrons from P700 to $P^{+}700$ through electron acceptors (Shiozawa et al,,1974).

Native pigment-protein complex II accounts for 40% of the total lamellae protein and has a molecular weight of between 21,000 and 35,000 daltons (Eaglesham and Ellis, 1974; Hiller et al., 1973; Kung and Thornber, 1971). Chlorophylls a and b are present in equimolar quantities (Argyroudi-Akoyunoglou and Akoyunoglou, 1973), in the probable ratio of 6 moles/28,000g protein (Thornber, 1975). All chloroplast carotenoids, particularly β-carotene, lutein and neoxanthin, are associated with this complex (Ogawa et al., 1966; Thornber and Highkin, 1974), resulting in a chlorophyll carotenoid ratio of 3 - 7:1 (mole:mole).

Due to the large chlorophyll content of this chlorophyll-

protein complex (50% of the total in some plants) it is envisaged that its function is to provide an antennae pigment system which can distribute excitation energy between PS II and PS I (Thornber, 1975).

Fractions containing the PS II reaction centre have been isolated using digitonin (Wessels et al., 1973) and Triton (Ke et al., 1974) fractionation procedures. Chlorophyll a:b ratios have been detected as high as 28:1 suggesting that the chlorophyll b associated with PS II is confined to the chlorophyll-protein complex II as antennae pigment. In addition, the PS II fractions are rich in C550, P680 (currently suggested to be primary acceptor and donor to PS II) and Mn (Ke et al., 1974) and contain both β-carotene and lutein (Wessels et al., 1973). Both fractions have demonstrated light induced C550 reduction and cytochrome b559 photooxidation at 77°K.

The described chlorophyll-protein complexes do not contain all the chloroplast chlorophyll. That which is not bound in these complexes is generally found in the free pigment zone. This, however, is thought to originate from the dissociation of chlorophyll-protein complex II during electrophoresis (Thornber, 1967) or from a third pigment-protein complex which runs concurrently with complex II(Genge et al., 1974). Other chlorophyll-protein complexes identified in the past (Guignery et al., 1974;Herrmann and Meister, 1972; Hiller et al., 1974) are more likely to be polymers of the two major complexes.

3. MOLECULAR ARCHITECTURE OF THE CHLOROPLAST MEMBRANE

3.1 External surface

The most relevant information on the structure of the external surface of the chloroplast membrane has been obtained from negative staining and immunological studies. The predominant proteins of the external thylakoid surface are coupling factor and carboxydismutase, both of which are loosely surface bound and have been located immunologically (McCarty and Racker, 1966; Kannangara et al., 1970) and by negative staining (Moudrianakis et al., 1968; Murakami, 1968). In addition there are an important group of proteins which extend into the membrane to different extents, and are associated with chloroplast electron transport. Immunological studies have shown the location of the PS I electron acceptors, ferredoxin (Hiedemann-van Wyk and Kannangara, 1971), ferredoxin-$NADP^+$reductase (Berzborn, 1969) and FDS (Regitz and Oettmeier, 1972) to be on the outer thylakoid surface. By contrast, the electron donors of PS II, cytochrome f and plastocyanin, are thought to be located on the inner (loculus) thylakoid surface (Hauska et al., 1971; Racker et al., 1972; Sane and Hauska, 1972). Data obtained for the sites of PS II electron acceptors and donors suggest a similar situation to that of PS I (Junge and Ausländer, 1974 a and b; Fowler and Kok, 1974 a and b), although contrasting evidence is also strong (Radunz et al., 1971; Braun and Govindjee, 1972; Arntzen et al., 1974). The possible arrangement of the electron

transport proteins within the membrane is considered in section 5.

3.2 Hydrophobic membrane interior

The internal lamellae of the chloroplast can be readily isolated and purified and for this reason they were one of the first biological materials to be extensively investigated by electron microscopy. In addition a number of other techniques including polarisation and X-ray diffraction analysis have provided different kinds of information which, when integrated, strongly suggest that chloroplast lamellae are composed of discrete subunits.

The classical model of the cell membrane that emerged from permeability measurements, chemical analysis and electrical studies was formulated as a bimolecular lipid layer sandwiched between two protein layers (Danielli and Davson, 1935) and for many years it was assumed that the general features of this model were applicable to all cellular membranes. The early observation of Frey-Wyssling and Steinmann (1948), using polarisation optics on chloroplast lamellae, suggested that the lipids were not highly ordered, as would be expected with the Danielli-Davson model.

Following the initial experiments of Kreutz and Menke (1960 a and b) using X-ray diffraction techniques, it soon became apparent the lamellar membranes might be composed of protein subunits arranged in 'crystallites' (Kreutz, 1963). Further work by Kreutz (1966) showed that 16 protein subunits formed a crystallite.

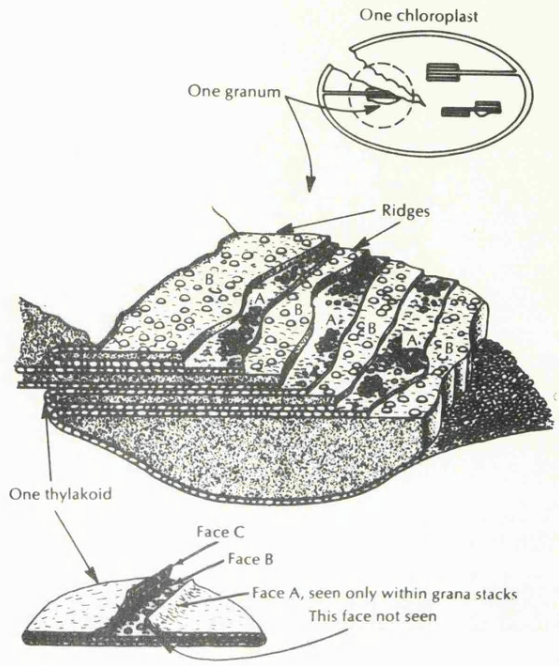
Shadowing techniques initially by Steinmann (1952) and later

by Park and Pon (1961; 1963) confirmed that the lamellar membrane was composed of particles. These were 155 x 180 x 100 Å in size and appeared to consist of several subunits (Park and Biggins, 1964).

Heavy metal positive staining of thin sections has shown the presence of transverse densities within lamellar membranes (Fuhs, 1966; Weier et al., 1965; Hohl and Hepton, 1965; Weier and Benson, 1967) which appear to be composed of four globular subunits approximately 90 Å in diameter. It is suggested that these latter particles are analagous to the crystallites of Kreutz and the 'quantasomes' of Park and co-workers (see section 5). It should be pointed out that all of the above mentioned studies of subunits in sectioned lamellae have been questioned and doubt cast upon their validity (Robertson, 1966). It is thought improbable that overlapping 90 Å globules can be visualised in a section which is itself 400 Å thick, as each particle would have to exactly align one above the other.

In recent years valuable information has been obtained from freeze-etched studies, a technique perfected by Moor (1964). This technique reveals not only the cross sections but also extended face views of the lamellar membranes (figure 5B)and has clearly demonstrated the presence of subunits within the membrane. The three different faces which are seen have been labelled to correspond with the model of Branton and Park(1967) illustrated in figure 5A. There is now general agreement that the fractures occur along the hydrophobic regions within the membranes, thus revealing matching internal hydrophobic faces B and C. The external thylakoid face A (see section 3.1) is also visualised by this method. The hydrophobic face of the internal

A



B

Figure 5. A: A model depicting Branton and Park's interpretation of thylakoid membrane ultrastructure (Branton and Park, 1967). B: Electron micrograph of spinach chloroplast thylakoids prepared by freeze etching. A, B and C represent the membrane faces shown in Figure A. Faces B and C contain the large (175Å x 90Å) and small (110Å x 90Å) particles respectively. Face A represents the external surface of the thylakoid but is seldom seen since the fracture generally occurs through the centre of the granum rather than along the external surfaces (Branton, 1968).

half of the membrane, B, is covered by particles of average diameter 175 Å and 90 Å thick. The particles are sometimes densely packed, occasionally occur in arrays, and frequently appear to be composed of three or four smaller subunits. The hydrophobic face of the external half of the membrane, C is covered by a smaller particle measuring 110 x 90 Å and appears to form part of an embedding matrix around the larger 175 Å units.

Recent experiments, with chloroplasts isolated from different material, have indicated that the Branton and Park model is only valid if the lamellae are derived from the partition regions of the grana stack. Stroma lamellae and end membranes of grana stacks, contain no large B-face particle (Remy, 1969; Phung-Nhu-Hung et al., 1970b; Sane et al., 1970). This distribution has been associated with a functional difference between stroma and grana lamellae (see section 5).

4. CHLOROPLAST FUNCTION

Photosynthetic electron transport in chloroplasts yields oxygen and NADPH, and is coupled to ATP formation. The process is localised in the inner lamellae system, and the products of this light reaction (ATP and NADPH) are utilised by the enzymes of the carbon dioxide assimilation cycle, localised in the stroma, to produce carbohydrates in a subsequent 'dark' reaction. Consequently for each mole of carbon dioxide that is reduced to the level of carbohydrates,

1 mole of oxygen is evolved and the net gain in free energy by the plant is 112,000 cals.

4.1 Two light reactions

The action spectrum for photosynthesis is broadly similar to the absorption spectra for the chlorophylls with absorption peaks in the red and blue regions of the spectrum. Discrepancies between the two spectra, however, are particularly large above 700 nm in the far-red region. Measurements of quantum yield of photosynthesis (Emerson and Lewis, 1942; 1943) were nearly constant for wavelengths less than 690 nm but dropped rapidly as the wavelength was increased to 710 nm. The low efficiency of far-red light can be increased by simultaneous illumination with shorter wavelength light, producing a rate of photosynthesis which is greater than that expected from the sum of the two incident beams alone (Emerson et al., 1957). The "Emerson enhancement effect" strongly suggested, therefore, that the co-operation of two light systems was required for maximum photosynthesis. The scheme represented in figure 6 is a recent and modified version of that proposed by Hill and Bendall (1960) to explain the accumulated data of Emerson and other workers.

4.2 Electron transport

According to this model a flow of electrons is initiated by the absorption of light quanta by the photosystems, hence the absorption

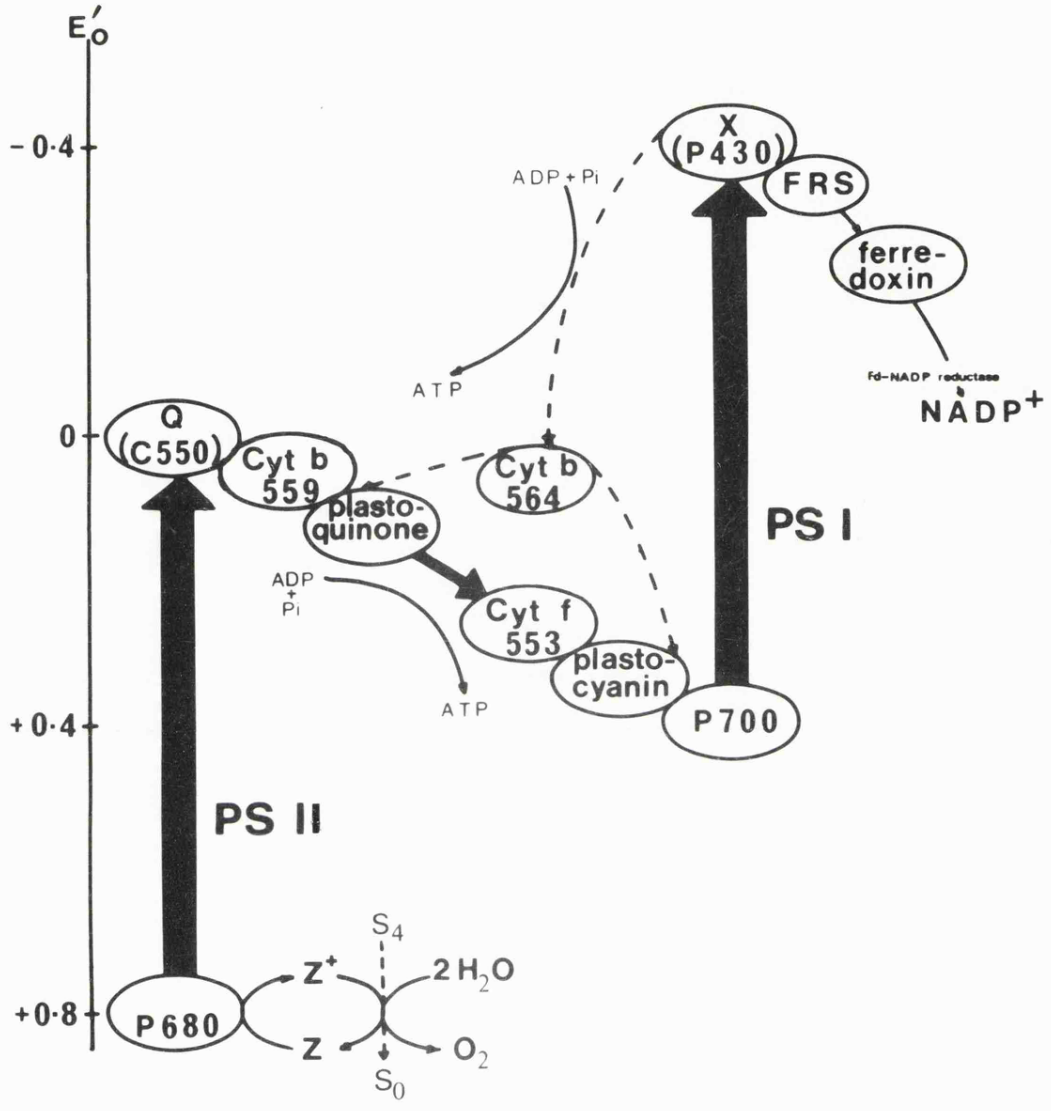


Figure 6. Z - scheme summarising current information on the electron transport path in photosynthesis. An overall flow of electrons from H_2O to $NADP^+$ is shown. S denotes the oxidoreducible water splitting enzyme. Z is the primary electron to the PS II reaction centre. P680 is the proposed energy trap for PS II. Q is the quencher of chl a fluorescence in PS II and is thought to be an unknown compound (C550) with a difference spectrum at 550 nm. Cyt b_{559}, plastoquinone, cyt f_{553} and plastocyanin are intermediate electron carriers. P700 is the probable energy trap for PS I. X is the primary electron acceptor for PS I, thought to be a pigment with one of its absorption bands at 630 nm. FRS is ferredoxin reducing substance. E'o is the oxidation reduction potential at pH 7 in volts.

of light by PS II results in the oxidation of water ($E'o = +0.80$) to free oxygen, and the reduction of Q ($E'o \simeq 0v$). Light absorbed by PS I oxidises P700 ($E'o = +0.40$) and reduces a low potential electron acceptor X ($E'o \simeq -0.6v$). The latter reduces ferredoxin, possibly via Ferredoxin Reducing Substance (FRS), which in turn donates electrons to $NADP^+$ in a ferredoxin-$NADP^+$reductase catalysed reaction.

Oxidised P700 is re-reduced by Q via a number of exergonic electron transport reactions which are coupled to the phosphorylation of ADP. In order of sequence the carriers catalysing the electron transport reactions are thought to be cytochrome b559 (low potential) plastoquinone, cytochrome f and plastocyanin.

The scheme represented in figure 6 requires that both photosystems are activated for complete photosynthesis. Light absorbed by PS II, however, can maintain an efficient rate of photosynthesis (Emerson et al., 1957), as excitation energy may be transferred from PS II to PS I. The control of excitation energy transfer is thought to operate through the redox state of P700. Excitation energy may only be passed onto PS I when P700 is in a natural unbleached state (Clayton, 1963) which can only occur when PS II is overdriven. More recently the control of excitation energy has been shown to be dependent on light induced fluxes in the metal cation composition of the thylakoids (Murata, 1970).

4.3 Photosystem II

4.3.1 Absorbing pigments

Although the chlorophylls are the predominant light absorbers other pigments are also present and function in either PS I, PS II or both. In general the short wavelength forms of chlorophyll a and both forms of chlorophyll b function as antennae light acceptors for the electron traps or reaction centres of PS II. In addition the xanthophylls are recognised to accept light in the PS II complex although excitation transfer between these and chlorophyll a is inefficient (see Govindjee, 1975).

4.3.2 Electron trap (P680)

Each light quantum absorbed by any pigment molecule of the photosystem is transferred through the complex until it reaches the specialised chlorophyll a molecules of the reaction centres or energy traps. The latter are thought to be closely associated with the electron mediators so that the light energy can be efficiently converted to chemical energy (see section 4.3.3).

Derivative and low temperature absorption changes (Döring et al., 1967; Floyd et al., 1971) have detected a fast absorbance change located within the predominant red absorption band of in vivo chlorophyll a. The spectrum of this absorbance change shows major bands at 682 and

435 nm with a minor band at 640 nm (Döring et al., 1969). The location of these bands on the action spectrum for PS II suggests that they may originate from the photoactive chlorophyll a that serves as the excitation energy trap of the PS II reaction centre. This specialised form of chlorophyll a was subsequently designated P680 (Floyd et al., 1971). P680 may function in two ways: It may be an oxidoreducible compound similar to P700 (see section 4.4) or it may be a sensitiser which when excited, enables Z to donate to Q directly (Döring et al., 1967; 1969).

4.3.3 Oxygen evolution

One of the least known links of the photosynthetic electron transport chain is the electron donor to the reaction centre of PS II. The entity is usually designated the symbol Z in the majority of schemes. It has been recognised spectrophotometrically by a light induced absorbance change at 320 nm (Vater et al., 1968) although this change has more recently been ascribed to plastoquinone (Stiehl and Witt, 1969).

Following excitation, P680 is thought to donate an electron to Q within 60 nsecs. (Mauzerall and Malley, 1971) and abstract an electron from Z. The second process is slower than the first, requiring approximately 20 μsec to reach completion (Mauzerall, 1972). Oxidised Z now accepts an electron from the oxidoreducible enzyme S, which can accumulate up to four oxidising equivalents. S is thought to exist in the forms or S_0, S_1, S_2, S_3, S_4 where the subscripts signify the

number of positive charges per molecule (Joliot et al., 1969; Kok et al., 1970; Forbush et al., 1971).

Reduced Q discharges its electron to the intermediate pool of oxidants (pool A) which is probably plastoquinone. This pool serves as an electron sink, while S_4 reacts with two molecules of water to release one molecule of oxygen:

$$Z\ chl\ Q \rightarrow Z\ chl^*\ Q$$

$$Z\ chl^*\ Q \rightarrow Z\ chl^+\ Q^-$$

$$Z\ chl^+\ Q^- \rightarrow Z^+\ chl\ Q^-$$

$$Z^+\ chl\ Q^- \rightarrow Z^+\ chl\ Q + A^-$$

$$Sn\text{-}1 + Z^+\ chl\ Q \rightarrow Sn + Z\ chl\ Q$$

$$S_4 + 2H_2O \rightarrow S_o + 4H^+ + O_2$$

Each of the four oxidant accumulation steps from S_o -- S_4 requires 1 quantum of light, and hence 4 quanta are required for the evolution of 1 mole of oxygen.

The donors Z and S have not yet been discriminated experimentally and hence it cannot be ruled out that they are identical. Strong evidence exists that Z and S are metalloproteins, more specifically ferro or manganoproteins. Manganese deficiency impairs the transfer of electrons from water to Q (Homann, 1968; Cheniae and Martin, 1970). Removal of manganese by washing with high concentrations of alkaline tris buffer or by heating results in the total loss of oxygen evolving capacity (Homann, 1967; Cheniae and Martin, 1966, 1970). Electron flow, however, can be maintained by the addition of DPC, suggesting

a primary functional role of manganese in the oxygen evolving reaction centre.

The high potential form of cytochrome b559 has been associated with the water splitting catalyst (S) at low temperatures and under conditions of stress (Knaff and Arnon, 1969a; Erixon and Butler, 1971; Erixon et al., 1972). Evidence from redox measurements and absorption studies suggest that this is not the case under normal physiological conditions, and the role of cytochrome b559 is more likely to be one of an intermediate carrier between PS II and cytochrome f (Levine and Gorman, 1966; Hind, 1968; Ben-Hayyin and Avron, 1970).

The primary electron acceptor (Q) also remains an enigma. It has been attributed to a light induced absorbance decrease at 550 nm and hence designated C550 (Knaff and Arnon, 1969b). The absorbance change is detectable at low temperature, suggesting C550 must function close to the primary photochemical conversion (Knaff and Arnon, 1969b), and also in the presence of DCMU which blocks electron transport just after Q (Duysens and Sweers, 1963). In spite of the accumulated information on the spectral properties of C550 and of its interactions with other electron carriers of the photosynthetic electron transport chain, the class of chemical compounds to which it belongs remains unknown.

4.4 Photosystem I

4.4.1 Light absorbing pigments

The long wavelength forms of chlorophyll a - 678, 685, 705

and 720 - are weakly fluorescent and predominantly present in PS I. In addition smaller amounts of chlorophyll b, the carotenes and possibly excitation energy ('spillover') from PS II contribute energy from the shorter wavelength light, to PS I.

4.4.2 P700

The primary electron donor and energy trap for PS I is associated with a light induced absorbance change at 700 nm (Kok, 1957). Corresponding absorption changes at 433 nm in the Soret region of the spectrum suggest that a specific chlorophyll a molecule, designated P700 (Kok, 1957), is responsible. The photooxidation of P700 occurs within 20 nsecs (Witt and Wolff, 1970) and is unaffected by temperatures as low as 150°C (Witt et al., 1961).

The electrons of photooxidised P700 are transferred ultimately to $NADP^+$ through ferredoxin. The process is catalysed by the flavo-protein ferredoxin-$NADP^+$reductase (Tagawa and Arnon, 1962). Some doubt exists as to the primary electron acceptor for P700, which until recently was ascribed to FRS (Yocum and San Pietro, 1969). This factor, although isolated in crude form, remains uncharacterised. Recently the primary electron acceptor P430 has been discussed (Hiyama and Ke, 1971).

The re-reduction of oxidised P700 can be blocked by the addition of DCMU, suggesting that water is the ultimate electron donor (Bishop, 1958). Although still a matter of contention the copper protein plastocyanin, has been placed as the immediate electron donor to P700 (Gorman and Levine, 1966; Hind, 1968; Avron and Shneyour, 1971),

accepting electrons itself from cytochrome f.

4.5 Photophosphorylation

Photophosphorylation in chloroplasts was first demonstrated by Arnon et al. (1954). In this process the high energy compound ATP is formed from ADP and Pi at site(s) in the electron transport chain where the energy, represented by a drop in redox, is converted to chemical energy.

4.5.1 Non-cyclic phosphorylation

Non-cyclic phosphorylation involves the normal electron flow and the co-operation of both photosystems, and the phosphorylation site(s) are thought to be in the intermediate electron transport chain between Q and P700. Promotion of phosphorylation in isolated chloroplasts by the addition of ADP and Pi also results in an increased electron flow from plastoquinone to cytochrome f. Similar observations were made when electron flow was uncoupled from phosphorylation by addition of NH_4Cl (Böhme and Cramer, 1972a). These data suggest that one site of photophosphorylation is between plastoquinone and cytochrome f.

4.5.2 Cyclic phosphorylation

The low potential electron acceptor (X) for PS I can either reduce $NADP^+$ or it can return its electron to an intermediate pool

carrier, probably cytochrome b6. The electrons reduce P700 via plastoquinone, cytochrome f and plastocyanin and are again cycled to cytochrome b6 through PS I. ATP formed during PS I mediated electron flow is referred to as cyclic phosphorylation and it has been suggested that the coupling site for the latter is also common for non-cyclic phosphorylation (Böhme and Cramer, 1972 b). ATP formation, during cyclic electron flow, is also thought to occur between X and cytochrome b6.

4.5.3 Mechanisms of photophosphorylation

At present there are two major competing mechanisms for phosphorylation. The Chemical Coupling hypothesis involves a covalent bond between an electron carrier with another entity designated X which is probably coupling factor. This step requires oxidation, which therefore moves the carrier couple from a low to high energy level (i.e. from -X to ~X). In a high energy state the carrier cannot act as an electron donor and therefore for electron transport to continue the carrier-X complex must be broken down by adding Pi and ADP across the bond to form ATP. The reaction may be summarized:

$$AH_2 + X \rightarrow AH_2\text{-}X$$

$$AH_2\text{-}X + B \rightarrow BH_2 + A\sim X$$

$$A\sim X + Pi \rightarrow X\text{-}Pi + A$$

$$X\text{-}Pi + ADP \rightarrow ATP + X$$

This latter mechanism does not explain phenomena of membrane electric potentials, ion fluxes and membrane structural changes, all of which are now known to be directly associated with the coupling mechanism.

The chemiosmotic hypothesis proposed by Mitchell (1961; 1968; 1970) is based on charge build up across a biological membrane, which is poorly permeable to protons, and which encloses a space. It assumes that a flow of reducing equivalents alternates between electron carriers, such as cytochromes, and hydrogen carriers such as plastoquinone, which occur on opposite sides of the membrane. When the reducing equivalents run from an electron to a hydrogen carrier, the extra proton is taken from the medium outside the chloroplast. The proton becomes unnecessary when the reducing equivalent moves back to an electron carrier and so is left inside the enclosed space. The positive charge on the inside of the membrane and the pH difference which is also created as a consequence produce a "Protonmotive force" (pmf) which is used to drive ATPase in the reverse direction.

5. STRUCTURE AND FUNCTION

5.1 Localisation and distribution of the photochemistry

Structural investigations using the electron microscope have revealed that virtually all green algae and higher plant chloroplasts contain distinct regions of lamellae appression, joined by single stroma lamellae. Initial chlorophyll fluorescence studies on the internal

lamellae led Spencer and Wildman (1962) to propose that total chloroplast chlorophyll was contained within the grana. This data was subsequently disproved by Lintilhac and Park (1966) who demonstrated that chlorophyll fluorescence could also be detected in the stroma lamellae.

Cytochemical staining using the reduction of silver nitrate (see Rabinowitch, 1956) and TNBT (Weier et al., 1966) has shown PS II activity to be located within the grana partitions. More recently Hall et al. (1971; 1972) have shown the location of PS II in both stroma and grana lamellae, although the authors point out that reduced ferricyanide in the stroma lamellae may be an artefact. Photochemical oxidation of DAB, however, has localised the presence of PS I over both stroma and grana and grana lamellae (Nir and Seligman, 1970; Chua, 1972).

Fractionation of the internal membrane into stroma and grana lamellae has produced the most direct means of determining the distribution of photochemistry within the two regions. Mechanical fractionation of chloroplasts by sonication (Gross and Packer, 1967) and French pressure cell procedures (Michel and Michel-Wolwertz, 1969) produced two fractions which were conclusively identified, as separate grana and stroma lamellae (Sane et al., 1970).

Biochemical studies on these fractions has shown them to be very different in both function and structure. The stroma lamellae fraction was observed to have a high chlorophyll a:b ratio (Gross and Packer, 1967) and greatly enriched in PS I activity (Michel and Michel-Wolwertz, 1969). The grana component, on the other hand, contained a lower chlorophyll a:b ratio but demonstrated both PS I and PS II

activity. Sane et al. (1970), using a modification of the French press procedure of Michel and Michel-Wolwertz, extended and refined the latter studies to conclude that, not only the stroma lamellae, but also the grana and membranes of mature intact spinach chloroplasts, contained only PS I activity, while the grana partition regions had both PS I and PS II. The arguments that followed suggested that these conclusions were based on artefacts produced by the deleterious effects of mechanical shearing forces. Subsequent experiments by Park and coworkers (Park and Sane, 1971; Park et al., 1971), however, provided data which suggests an absence rather than an inactivation of PS II within the stroma lamellae.

The use of low concentrations of detergents for lamellae fractionation also results in the release of stroma lamellae from intact grana (Goodchild and Park, 1971; Sane et al., 1970). Higher concentrations produced a secondary action of selective solubilisation and release of PS I from both grana and stroma lamellae (Arntzen et al., 1972; Vernon et al., 1967). These data have resulted in speculation as to the localisation of PS I and PS II within the membrane. Anderson and Boardman (1966) proposed that PS I was loosely attached to the membrane whereas PS II was intrinsically bound. Freeze-etch examination of the digitonin fraction (Arntzen et al., 1969; 1972) has shown that the PS I and PS II active fractions contained the small C-face and large B-face particles respectively. It was concluded from this data that detergent action caused longitudinal cleavage along the hydrophobic interior of the lamellae in a manner similar to the freeze fracture process (see section 3.2). The action of detergents therefore appears

to solubilise PS I subunits from the surface of the membrane leaving the portion of the membrane containing PS II.

In addition to the above data, site specific labelling with antibodies (see section 3.1) and with (^{35}S) diazonium-benzenesulphonic acid (Dilley et al., 1972) have both presented data which is consistent with the concept of an asymetric distribution of functional components, with PS I being exposed at the surface of the membrane.

5.2 Development of chloroplast function and structure

Membrane biosynthesis which occurs during chloroplast development, has become generally accepted to be a multistep assembly process. The development of the higher plant chloroplast lamellar system (see Introduction section 2.3) has been studied by placing etiolated plants in continuous or flashing light regimes, and then following both biochemical and morphological changes.

The initial studies of Anderson and Boardman (1964) demonstrated that PS II-mediated ferricyanide reduction could be detected in chloroplasts from dark grown bean leaves after 5h illumination. $NADP^+$ photoreduction was not observed until after 8h illumination and this did not reach a half maximal rate until after 16h. Recently Boardman et al., (1970) demonstrated the appearance of PS I activity, as measured by the photooxidation of cytochrome f, after only 30 min. It appears, therefore, that the onset of PS I preceeds that of PS II during the developmental sequence of membrane biosynthesis. This data also provides evidence as do other results (Bradbeer et al., 1969;

Gyldenholm and Watley, 1968), that the enzymatic apparatus for transferring electrons from PS I to $NADP^+$ may be the last part of the electron transport system to be developed. Comparative structural studies using electronmicroscopy (Boardman and Anderson, 1964; Boardman et al., 1970) correlated the appearance of grana stacks with the first detection of PS II activity.

Studies on the onset of photophosphorylation by Dodge and Whittingham (1966) and Gyldenholm and Whatley (1968) have shown that cyclic phosphorylation commences at approximately the same time as PS I (5h) activity and preceeds the onset of noncyclic phosphorylation. Analysis of the greening tissue (Gyldenholm and Whatley, 1968) again showed that more extensive grana stacking correlated with non-cyclic photophosphorylation, which in turn required a functional PS II. Rhodes and Yemm (1966) and Miller and Noble (1972) found a correlation between the onset of grana formation and the ability to fix CO_2 in greening barley chloroplasts. Further studies by Phung-Nhu-Hung et al. (1970b) also using barley have shown a pattern of results similar to those of previous workers. The presence of PMS catalysed cyclic phosphorylation and non-cyclic phosphorylation as measured by $NADP^+$ reduction were, however, demonstrated somewhat earlier at 4 and 10h illumination respectively. Grana stacking was observed after 4h illumination and therefore independently of PS I activity.

Numerous functional and structural studies have been conducted on chloroplasts which have been isolated from leaves greened under intermittant illumination of 1 ms flashes separated by 15 min dark intervals (Sironval et al., 1968b; Phung-Nhu-Hung et al., 1970a). These have shown the presence of long unpaired primary thylakoids

which have a high chlorophyll a:b ratio, an active PS I mediated cyclic phosphorylation activity, but no detectable PS II activity and a deficiency in cytochrome b559. More recently Henningsen and Boardman (see Arntzen and Briantais, 1975) have shown that oxygen evolution in greening barley occurred ahead of the formation of high potential cytochrome b559. Hiller and Boardman (1971) observed a correlation in the presence of high potential cytochrome b559 with grana stacking and which appeared to be independent of PS II activity. Other studies on greening under intermittant light (Argyroudi-Akoyunoglou and Akoyunoglou, 1970) and in far-red light (Oelze-Karow and Butler, 1971; De Greef et al., 1971) have shown selective chlorophyll a accumulation in unpaired lamellae, together with high PS II activities.

These correlative functional and structural studies on chloroplast development have shown that the relationship between PS II and grana formation is a complex one. A more detailed survey on present ideas as to the physiological role of grana stacking is presented in Discussion section 1.

5.3 The Quantasome

Emerson and Arnold (1932), on the basis of flashing light experiments, proposed a functional photosynthetic unit containing 2,500 chlorophyll molecules which could fix 1 mole of carbon dioxide for every mole of oxygen evolved. In order to allow for the transfer of four electrons per mole oxygen, each of which requires two photosystems, the number of chlorophyll molecules contained in the PS II

was later amended to 250 - 300 (Kohn, 1936).

The detailed chemical analysis by Park and Pon (1963) on spinach chloroplast lamellae produced data which suggested that such a unit could exist. The unit was designated a "quantasome" and was subsequently identified by negative staining techniques (Park and Biggins, 1964). In initial freeze-etch studies Branton and Park (1967) demonstrated that the quantasome seen by negative staining was identical to the large 175 Å particle together with matrix material from the membrane. Removal of the matrix material left what was referred to as a quantasome core (Park and Sane, 1971).

Membrane fractionation procedures to isolate an individual functional quantasome have failed although Gross et al. (1964) isolated aggregates of 2 - 3 quantasomes which were found to have fully active Hill activities. Izawa and Good (1965) pointed out, however, that the harsh methods of isolation caused uncoupling. Acknowledgement of this factor in subsequent experiments showed the quantasomes to be almost completely photochemically inactive.

Recent investigations with detergent fractionation have shown that the PS I fraction contained only small freeze-etch particles. The quantasome core, the large freeze fracture particle, was PS II enriched, but contained no PS I components (Arntzen et al., 1972). In addition, present evidence suggests that the large and small freeze-etch particles are markers for PS I and PS II respectively, but do not represent functional units. Normal photosynthetic capabilities have been demonstrated in two mutants of Chlamydomonas reinhardii, which are

agranal and contain no large freeze fracture particle (Goodenough and Staehelin, 1971). The appearance of the 175 Å particle was induced, however, by high salt concentrations, without affecting the photochemical activities.

6. CONCLUDING SECTION

The preceding sections of this Introduction have outlined the problems which occur when correlating function and structure during chloroplast development. The majority of investigators have attempted to find the basic requirements for the instigation of functional activity in developing chloroplasts, and in some instances to relate these to structural changes which occur on the same time scale. This method of approach has proved to be relatively successful as shown in section 5.2, however major problems remain because of attempts to correlate results from different plant species, or from similar plants treated in a different manner. In addition there are major problems of interpretation and correlation of electron microscopic data. A particular example is the correlation of the instigation of PS II activity with grana stacking (Anderson and Boardman, 1964; Rhodes and Yemm, 1966). For almost ten years this has remained a point of contention, and it is now becoming apparent that the relationship between the two may be purely coincidental (see Discussion section 1).

An approach taken by Klein and Neuman (1966) and Schiff et al. (1967) and used in this present study, has been to determine how

far chloroplast development is dependent upon functional activity, by greening leaves in the presence of a photosynthetic inhibitor. The herbicide, CMU, was found to be a potent inhibitor of electron transport in isolated chloroplasts (Wessels and Van Der Veen, 1956) and it is well known that it leads to the cessation of photosynthetic CO_2 uptake in whole leaves (van Oorschot, 1965). The site of action of CMU and related herbicides (see Dodge, 1975) is shown in figure 8. Klein and Neuman (1966) greened etiolated bean leaves in the presence of CMU and showed that the development of chloroplast fine structure and chlorophyll synthesis was markedly inhibited. However, the effect could be reversed by the addition of exogenous sucrose, to produce green leaves with chloroplasts which were morphologically similar to the control. Schiff et al. (1967) greened Euglena in the presence of the related herbicide DCMU and concluded that photosynthetic competence was not necessary for chloroplast development.

In this present study a detailed investigation has been made of the development of various aspects of the electron transport system of pea chloroplasts isolated from both photosynthetically competent and incompetent plants. The onset and development of PS II has been measured using both silicomolybdate and ferricyanide which accept electrons from two different sites in the chain before and after the CMU site of action. In addition, whole chain electron transport has been measured using paraquat as terminal acceptor for PS I. The uptake of manganese and its incorporation into lamellae protein has been used as a measure of the development of the PS II reaction centres and associated oxygen evolving mechanism. Similarly the

instigation and development of PS I has been measured by both the photooxidation of ascorbate and the reduction of $NADP^+$ from the ascorbate/DCIP redox couple. Using this approach it has been possible to isolate small sections of the electron transport system and to study their development with respect to the total. Additional information into the development of the photosystems has been made by a comprehensive study of the changes occurring in the carotenoid and chlorophyll contents during greening.

Structural studies have also been undertaken to both complement and supplement the information obtained from the photochemical data. The role of the chlorophyll-protein complexes as structural and functional entities of the photosynthetic membrane has been discussed in section 2 of the Introduction. The synthesis and insertion of two of these complexes into the developing membrane has been monitored by SDS polyacrylamide gel electrophoresis. Furthermore a comprehensive study of changes in chloroplast fine structure, occurring after the onset of illumination, have been conducted by electronmicroscopy. In conclusion, therefore, it has not only been possible to compare chloroplast development in functionally competent and incompetent plants, but also to correlate changes occurring in the electron transport system with observed structural changes.

MATERIALS AND METHODS

MATERIALS AND METHODS

1. TREATMENT OF PLANT MATERIAL

Pea seeds (Pisum sativum var. Meteor) were grown on Levington compost for six days at 25°C in continuous darkness. Etiolated shoots were detached from the cotyledons 5 cm below the apex and placed in vials (ten per vial) containing 10 ml of sucrose (5×10^{-2}M) and CMU (5×10^{-4}M) as required. The explants were incubated in darkness for 16 h, prior to illumination with white light of 6 W/m^2 at a temperature of 22.5°C and 70% humidity. All experimental manipulations before illumination were carried out under a green safe light (Kodak Ltd.).

CMU solutions(1×10^{-3}M) were prepared by dissolving the solid in methanol (2% of final volume). Water was then added and the resultant suspension was refluxed until a clear solution was obtained (3 - 5 h). Fresh solutions were prepared weekly and so far as could be ascertained no loss in CMU activity was detected over this period.

2. CHLOROPLAST ISOLATION PROCEDURES

All experimental manipulations were carried out at 4°C, unless otherwise stated.

2.1 Chloroplasts for electron transport assays - (Method of Izawa and Good, 1968)

Pea leaves (5 g) were homogenised in a mortar containing a grinding medium (20 ml) which consisted of tricine buffer (40 mM) pH 7.5, NaCl (300 mM) and $MgCl_2$ (2 mM). The homogenate was squeezed through four layers of muslin and the filtrate centrifuged at 1,200g for 1 min to remove sand and cell debris. The supernatant was centrifuged at 3,000g for 10 min and the resultant chloroplast pellet resuspended in 10 ml tricine buffer (30 mM) pH 7.3 containing sucrose (200 mM) and $MgCl_2$ (3 mM). The chloroplast suspension was centrifuged at 3,000g for 10 min and the pellet resuspended in 2 ml of the latter buffer.

2.2 Chloroplast lamellae for manganese determination - (Method of Alberte, Thornber and Naylor, 1972)

Pea leaves (5 g) were homogenised in a mortar containing tris buffer (50 mM) pH 8.0, $MgCl_2$ (2 mM) and a little sand. The homogenate was filtered through four layers of muslin and the filtrate centrifuged at 10,000g for 1 min to remove sand and cell debris. The supernatant was centrifuged at 20,000g for 10 min and the resultant pellet resuspended in the above buffer (10 ml). The lamellae were washed to remove soluble protein by centrifuging twice at 40,000g for 10 min. The final pellet was resuspended in the initial buffer (2 ml).

2.3 Chloroplasts for polyacrylamide gel electrophoresis

Pea leaves (5 g) were macerated for 10s in 35 ml of tricine buffer (25 mM) containing sucrose (350 mM), EDTA (2 mM) and mercaptoethanol (20 mM). The homogenate was squeezed through four layers of muslin and the filtrate centrifuged at 1,500g for 30s to remove cell debris. The supernatant was recentrifuged at 10,000g for 10 min. The pellet was washed by resuspending in 20 ml tris-glycine buffer (20 mM) containing mercaptoethanol (10 mM) and glycine (4 mM) and centrifuging twice at 30,000g for 10 min. These final washing steps were carried out to remove Fraction I protein. The final pellet was resuspended in the washing buffer (1 ml).

3. ASSAY SYSTEMS FOR CHLOROPLAST ACTIVITY

3.1 Oxygen electrode

Reactions carried out in an oxygen electrode (Rank Bros.), routinely consisted of 3 ml of experimental solution at a constant temperature of 20°C. Illumination was provided by 1,000 W lamp giving 280 W/m^2 at the reaction chamber. The electrode was calibrated before each experiment and the results calculated from the initial rates of oxygen exchange.

The reagents used in each of the assays were as follows:-

3.1.1 PS II (ferricyanide reduction)

Tris buffer	(0.3M) pH 8.0	- 0.3 ml
Potassium ferricyanide		- 0.2 ml
Chloroplasts	(150µg chl where possible)	
Water		to 3 ml

3.1.2 PS II (silicomolybdate reduction)

Tris buffer	(0.3M) pH 8.0	- 0.3 ml
Silicomolybdate	(10mg/ml)	- 0.1 ml
Chloroplasts	(150µg chl where possible)	
Water		to 3 ml

3.1.3 PS I and PS II

Tricine buffer	0.200M)	
	)	
NaH_2PO_4	0.002M)- pH 8.0	- 1.6 ml
	)	
NaN_3	0.002M)	
Paraquat	(1×10^{-4}M)	- 0.1 ml
Chloroplasts	(150µg chl where possible)	
Water		to 3 ml

3.1.4 Ascorbate photo-oxidation (PS I activity)

Tris buffer	(0.3M) pH 8.0	- 0.3 ml
DCIP	(1×10^{-3}M)	- 0.2 ml
Ascorbate	(0.2M)	- 0.2 ml
Paraquat	(1×10^{-4}M)	- 0.1 ml
Chloroplasts	(80µg chl where possible)	
Water		to 3 ml

3.2 Spectrophotometric assays

3.2.1 Ferredoxin mediated $NADP^+$ reduction

This reaction was carried out in 1 cm light path quartz cuvettes containing:-

Tris buffer	(0.3M) pH 8.0	- 0.30 ml
Ascorbate	(0.2M)	- 0.20 ml
$NADP^+$	(2.5µmol/ml)	- 0.20 ml
DCIP	(1×10^{-3}M)	- 0.05 ml
CMU	(1×10^{-3}M)	- 0.10 ml
Ferredoxin	(2 mg/ml)	- 0.05 ml
Chloroplasts		- 0.30 ml
Water		- 1.80 ml

A reagent blank, containing no ferredoxin, was prepared and treated identically to the reaction cuvette. This was used to zero the

spectrophotometer (Unicam SP 1800) prior to each reading.

Illumination was provided by a 1,000 W lamp and the ΔOD_{340} taken after measured time intervals. Results were calculated on the basis if the extinction coefficient: ΔE 0.1 = 0.48μmols NADPH.

3.3 PMS mediated cyclic phosphorylation

Cyclic phosphorylation experiments were carried out in 10 ml Tünberg tubes at 20°C, containing 3 ml of a standard reaction mixture:-

Tricine buffer	0.20M)	
	)	
NaCl	0.02M)- pH 8.0	- 1 ml
	)	
Na_2HPO_4	0.01M)	
$MgCl_2$	0.10M	- 0.20 ml
PMS	(5×10^{-3}M)	- 0.02 ml
ADP	(0.1 mmol/ml)	- 0.05 ml
Glucose	(0.1M)	- 0.20 ml
Hexokinase	(10 mg/ml)	- 0.05 ml
Chloroplasts	(150μg chl where possible)	
Water		to 3 ml

Dark control tubes (wrapped in silver foil) were prepared for each treatment and treated identically to the reaction tubes. These were placed into a constant-temperature waterbath, evenly illuminated from the base by 8 x 275 W photoflood bulbs (Philips),

producing 275 W/m^2 at the reaction tubes. The tubes were constantly shaken during the experiment. Reactions were terminated after a measured time period by the addition of 0.3 ml TCA (11.5%) and the precipitate centrifuged at 2,500g for 5 min. 20µl of the supernatant was diluted to 0.9 ml for the phosphate assay (see methods 5.2)

The amount of phosphate esterified into ATP was determined by the difference in content between the illuminated tubes and a dark control.

4. CO_2 EXCHANGE IN PLANT MATERIAL

The CO_2 exchange of leaf tissue was measured by using an IRGA (Grubbs Parson) linked to an open circuit gas flow system (see figure 7). Air from a cylinder was dried in a series of calcium chloride-containing towers. The air line was then separated into two streams, one of which was coupled directly to the IRGA reference tube. The second air line passed through a water tower prior to the sample chamber to prevent plant dessication during longer term experiments. Water vapour was removed after the experimental chamber by incorporating another drying tube.

Flowmeters (G. A. Patron) ensured an identical air flow (500 ml/min) through both sample and reference tubes of the IRGA.

The sample chamber was illuminated by a photoflood lamp

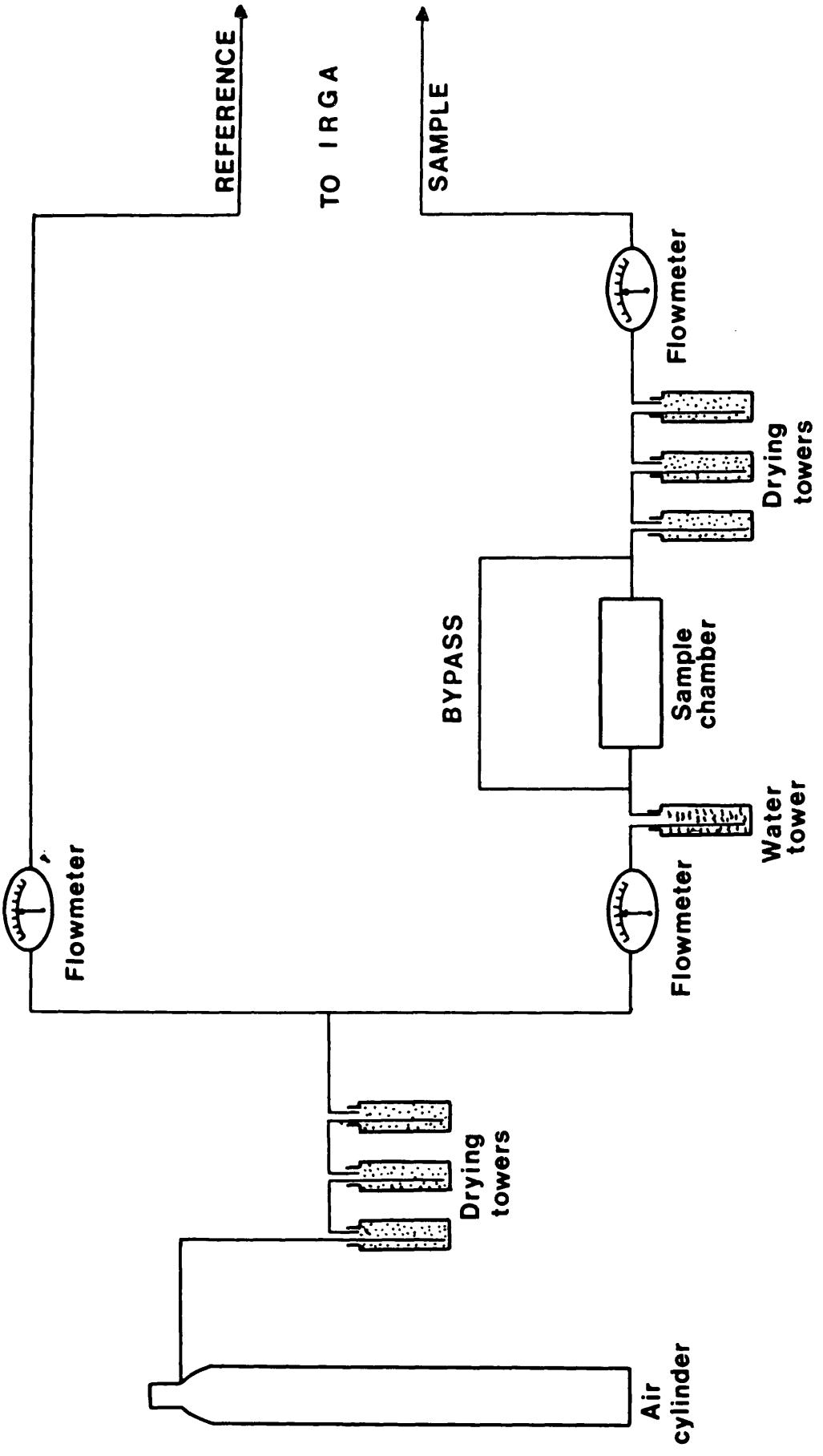


Figure 7. Diagram showing the gas flow circuit of the Infra Red Gas Analyser apparatus.

producing 115 W/m^2 at its upper surface. Heat dispersal was effected by passing the light through a glass screen containing constantly circulating 1% $CuSO_4$ solution at 20°C.

The rate of CO_2 uptake was calculated from:-

$$y = xCf/M \times 10^6$$

Where- y = rate of CO_2 uptake in ml/min

x = difference between light and dark traces

M = Recorder/IRGA magnification

C = Calibration of one IRGA division

5. QUANTITATIVE DETERMINATIONS

5.1 Protein determinations - (Method of Lowry et al, 1957 as modified by Hartree, 1972).

SOLUTION A

Potassium sodium tartrate	- 2 g
Sodium citrate ? carbonate	- 100 g
Sodium hydroxide (1N)	- 500 ml
Water	- to 1 litre

SOLUTION B

Potassium sodium tartrate	- 2 g
Copper sulphate	- 1 g
Water	- 90 ml
NaOH (1N)	- 10 ml

SOLUTION C

1 volume of Folin-Ciocalteu reagent

15 volumes of water

Solution A(0.9 ml) was added to suitably diluted solubilised lamellae protein (1 ml) and incubated at 50°C for 10 min. The solution was cooled to room temperature and 0.1 ml solution B added. After 10 min, 3 ml of solution C was injected to ensure rapid mixing within 1s. The colour was developed at 50°C for 10 min and then cooled to room temperature before reading at 650 nm in glass cuvettes of 1 cm light path.

The assay was found to be sensitive to 10µg protein and the calibration curve was linear to 120µg protein.

5.2 Phosphate determination - (Method of Ames, 1966)

SOLUTION A

Ascorbic acid - 10% w/v - prepared freshly

SOLUTION B

Ammonium molybdate (0.42% w/v) in 1N H_2SO_4 (28.6 ml/litre)

Solution A (1 vol.) was mixed with solution B (6 vol.) and stored in an ice bath during the assay. 2.1 ml of this mixture was added to 0.9 ml of phosphate solution and incubated at 45°C for 20 min. The solution was cooled to room temperature prior to reading at 820 nm in 1 cm glass cuvettes. The phosphate calibration curve was found to be linear between 20 and 100 µmols.

5.3 Chlorophyll determination

5.3.1 Leaf tissue

Whole leaves (0.1 g) were ground under a green safety light, in a mortar containing a small amount of 80% acetone and a little sand. Grinding was continued as the acetone volume was increased to 4 ml. The precipitate was centrifuged at 2,500g for 5 min and the resultant supernatant volume increased to 5 ml. Samples were read at 663 and 645 nm to determine total chlorophyll and the levels of chlorophyll a and chlorophyll b in accordance with the method of Arnon (1949).

5.3.2 Chloroplast suspensions

4 ml of 80% acetone was added to 5 ml of chloroplast suspension and the precipitate centrifuged at 2,500g for 5 min. The supernatant

was made up to a known volume (5 ml) and the chlorophyll concentration determined as in section 5.3.1.

5.4 Carotenoid estimation - (based on the method of Bishop and Wong, 1971)

Total pigments were extracted from 1 g plant material by grinding in a mortar containing a little acetone and sand. The acetone volume was increased during grinding to 30 ml and the precipitate was centrifuged at 2,500g for 5 min. The extract was placed in the dark and the pellet re-extracted with a further 10 ml acetone.

The bulk extract was partitioned into ether (30 ml) with the aid of saturated NaCl solution (100 ml). The ether extract was taken to dryness under vacuum and redissolved in 3 ml of ether. 1 ml of this extract was streaked onto a 10 x 10 cm silica gel T.L.C. plate, and developed in Petroleum ether: isopropyl alcohol: water (100:10:0.5), for 2 h.

Six carotenoid bands were resolved by this method. Combined α and β-carotene, and lutein and violoxanthin bands were quickly removed and eluted in petroleum ether (carotenes) or ethanol (xanthopylls). Quantitative estimations were carried out using the extinction coefficient of the predominant carotenoid (Davies, 1965).

5.5 Manganese determination

Double glass distilled deionised water was used in subsequent solutions and dilutions unless otherwise stated.

DIGESTION ACID

Sulphuric acid - 30% w/v

Perchloric acid - 28% w/v

Sodium molybdate - 2% w/v

10 g sodium molybdate (Analar) were dissolved in 150 ml water. Using an ice bath 150 ml sulphuric acid (Aristar) was added to the sodium molybdate solution with constant stirring. When the solution was completely cool 200 ml of 70% perchloric acid (Aristar) was added.

5.5.1 Whole tissue

A known weight of plant tissue was ashed overnight in a muffle furnace at 400°C. The white ash was dissolved in 1 ml of digestion acid and diluted with water to give an acid content of not more than 5%.

5.5.2 Chloroplast suspension

1.5 ml chloroplast lamellae suspension (see methods 2.2) was pipetted into a small Kjeldahl digestion tube containing one antibumping

granule and 1 ml digestion acid. A reagent blank (no lamellae suspension) was treated in an identical manner. The samples were hydrolysed by boiling on an electric Kjeldahl apparatus (M.S.E.), until all water was removed and the solutions were clear. The sample volumes were increased to 5 ml with water and the manganese contents assayed on an atomic absorption spectrophotometer (Perkin-Elmer) at a wavelength of 247.48 nm.

The manganese contents were expressed in relation to the lamellae protein content.

6. PREPARATION OF FERREDOXIN

Ferredoxin was isolated from pea leaves using the basic method of Tagawa and Arnon (1962) as modified by Kenesztes-Nagy and Margoliash (1966).

Fresh pea plants (1 kg) were cut, packed in plastic bags and frozen overnight at -15°C. The frozen material was minced and added with occasional stirring to 3l of distilled water containing 1 g solid Tris. The debris was removed from the homogenate by filtering through a funnel covered with a double layer of muslin and fitted with a glass wool plug.

A sufficient amount of NaCl was added to the filtrate to increase the concentration to 0.15M and the solution was passed through a column containing a bed of DEAE cellulose (16cm diam x 3cm high) previously equilibrated with a solution containing 10mM Tris buffer,

pH 7.5, and 0.15M NaCl. Green particulate matter, trapped in the resin during the adsorption step, was dispersed by repeatedly suspending the resin in 500 ml of the above solution and washing under suction.

Ferredoxin (approx.100 ml) was eluted from the column with a solution containing 10mM Tris buffer pH 7.5 and 0.8M NaCl. Particulate matter in the eluate was removed by centrifugation at 10,000g for 30 min. The ferredoxin was concentrated to approximately 20 ml by passing it through a smaller DEAE cellulose column and eluting off with 10mM Tris buffer, pH 7.5 and 0.8M NaCl.

The dark red-brown eluate was passed through a sephadex G.25 column (2cm diam x 20cm high; Pharmacia) previously equilibrated with 20mM Tris buffer pH 7.5. The excluded ferredoxin fraction was finally concentrated to approximately 10 ml on a DEAE cellulose column (1.5cm diam x 3cm high), eluted with a solution of 10mM Tris buffer pH 7.5 and 0.8M NaCl and stored under N_2 at -15°C.

7. SDS POLYACRYLAMIDE GEL ELECTROPHORESIS - (Based on the unpublished methods of Ellis)

Gel preparation:

Solution A	Tris buffer pH 8.5	- 36.3% w/v
Solution B	SDS	- 0.8% w/v
	Urea	
Solution C	acrylamide	- 40.0% w/v
	bis-acrylamide	- 0.8% w/v

Solution D Ammonium persulphate - 0.75% w/v

Solution E TEMED - concentrated

% gel	SOLUTIONS (ml) A	B	C	D	E
10	5	5	10	20	0.05
15	5	5	15	15	0.05

Dialysis (Viscing) tubing was wetted, cut along the seam and opened out into a single membrane sheet. Squares (approx. 2cm x 2cm) were cut and placed over the rubber grommets of the gel holder apparatus. The gel tubes (10cm x 0.8cm) were pressed into the grommets so that they were sealed by the dialysis tubing and held in an upright position. Solutions A to E were mixed to give the required gel percentage and 1.2 ml was pipetted into each of the tubes which had previously been treated with dichlorodimethylsilane (Fisons) to assist final removal. Water was carefully layered onto the surface of the gel solution to aid polymerisation and to ensure a flat loading surface. The gels were incubated at 30°C until polymerisation was complete (45 min).

Polymerised gels were pre-electrophoresed prior to loading at 10mM per gel for 1 h. The running buffer consisted of:-

Tris	28.8g	)
Glycine	6.0g	) pH 8.5
SDS	1.0g	)

Loading gels:

Isolated chloroplasts (see methods 2.3) were solubilised by adding 20% SDS in an SDS:protein ratio of 10:1 w/w. The extract was centrifuged at 2,500g for 5 min to remove any unsolubilised material and densified with solid sucrose. Chloroplast lamellae containing 50µg protein were loaded onto the gel by a syringe through the top running buffer. Electrophoresis at 5mA per gel was carried out for 2 h in a Shandon apparatus.

Visualisation:

a) Unstained gels.

Gels were scanned unstained at 660nm (Unicam SP 1800 - SP 1809 densitometer attachment) for chlorophyll containing bands. Absorption spectra of the chlorophyll contained in these were obtained by stopping the gel carriage and scanning each band between 380 - 700nm.

b) Staining.

The gels were removed from their tubes and stained overnight in coomassie blue R.250 (0.1%), ethanol (50%) and acetic acid (7%). Excess dye was leached out of the gels into ethanol (50%) and acetic acid (7%) over a period of 6 days, with regular changing. Densitometer scans of the protein bands were obtained by scanning the gel length in an SP 1809 densitometer attachment.

8. ELECTRON MICROSCOPY

Tissue processing.

REAGENTS.

A. Phosphate buffer pH 7.4

$Na_2HPO_4.2H_2O$ (3.56% w/v)	81.0 ml
$NaH_2PO_4.2H_2O$ (3.12% w/v)	19.0 ml

B. Glutaraldehyde fixative

Phosphate buffer pH 7.4	10.0 ml
Glutaraldehyde (25%)	2.4 ml
Water	7.6 ml

C. Washing medium

Phosphate buffer pH 7.4	25.0 ml
Sucrose	25.0 ml
Water	5.0 ml

D. Osmium fixative

Osmium tetroxide (2%)	5.0 ml
Phosphate buffer pH 7.4	5.0 ml

E. Alcohol

For dehydration of tissues 30, 50, 70, 90 and 100% alcohols were prepared.

F. Embedding medium (Standard Spur)

Vinyl cyclohexane dioxide (ERL - 4206)	10.0g
Dilysidyl ether (glycol polypropylene) (DER 735)	6.0g
Nonamyl succinic anhydride (NSA)	26.0g
Dimethyl amino ethanol (S-1)	0.4g

Procedure.

Longitudinal leaf sections (2mm x 1mm) were taken and placed in 2 ml glutaraldehyde fixative for 2 h. If required, a little alcohol was added to wet the leaf surface and enhance fixative impregnation. The fixative was removed and replaced by washing medium. All traces of glutaraldehyde were removed over a period of 24 h with 2 - 3 changes of washing medium.

Following the washing sequence the sections were post fixed with osmium tetroxide for 1.5 - 2 h until dark brown or black. The material was washed again for 15 min in the sucrose washing medium.

The sections were passed through a series of increasing alcohol concentrations; 10 min in each. Three changes of 100% alcohol were made, the final one lasting 20 min.

At the end of the dehydration sequence, half of the alcohol was removed and replaced by the same volume of Spur (i.e. Alcohol: Spur = 50: 50). The tissue was impregnated in this solution for 90 min, then half of the solution was removed and replaced by Spur (Alcohol: Spur = 25: 75). After 2 h the Spur-alcohol mixture was removed, replaced by 100% Spur and left overnight in a rotary stirrer. The material was subsequently embedded in Spur and incubated at 60°C until hard.

The resin blocks were trimmed, sectioned on an ultramicrotome (LKB) and the sections mounted on copper grids.

Staining procedure.

Reagents:-

A. Uranyl acetate - saturated in 50% alcohol (stored in darkness).

B. Renolds lead citrate (stored CO_2 free).

N.B. Stains are always centrifuged before use.

A few drops of uranyl acetate were placed on a square of dentist's moulding wax contained in a petri dish (diameter 4 cm). The wax was surrounded by 50% alcohol to maintain the correct atmosphere for the reagent. The copper grids were placed, section side down, on the drops. This procedure was carried out quickly so that the stain was not subjected to unnecessary light. The lid was replaced, the dish returned to darkness. After 30 min the grids were removed carefully with forceps, washed once in 50% alcohol and twice with distilled water. After blotting dry the grids were placed on drops of Renolds lead citrate on wax in a second petri dish. The atmosphere of this petri dish was maintained CO_2 free using wetted sodium hydroxide pellets around the square of wax. Care was taken to avoid contact with exhaled CO_2 which would precipitate lead in the section. The petri dish was covered and the sections stained for 30 min. The grids were then removed, washed once in 0.02% NaOH, twice in distilled water, and finally blotted dry. The sections were fully prepared for inspection under the electron microscope (Philips) and data was recorded on 35 mm Kodak safety film.

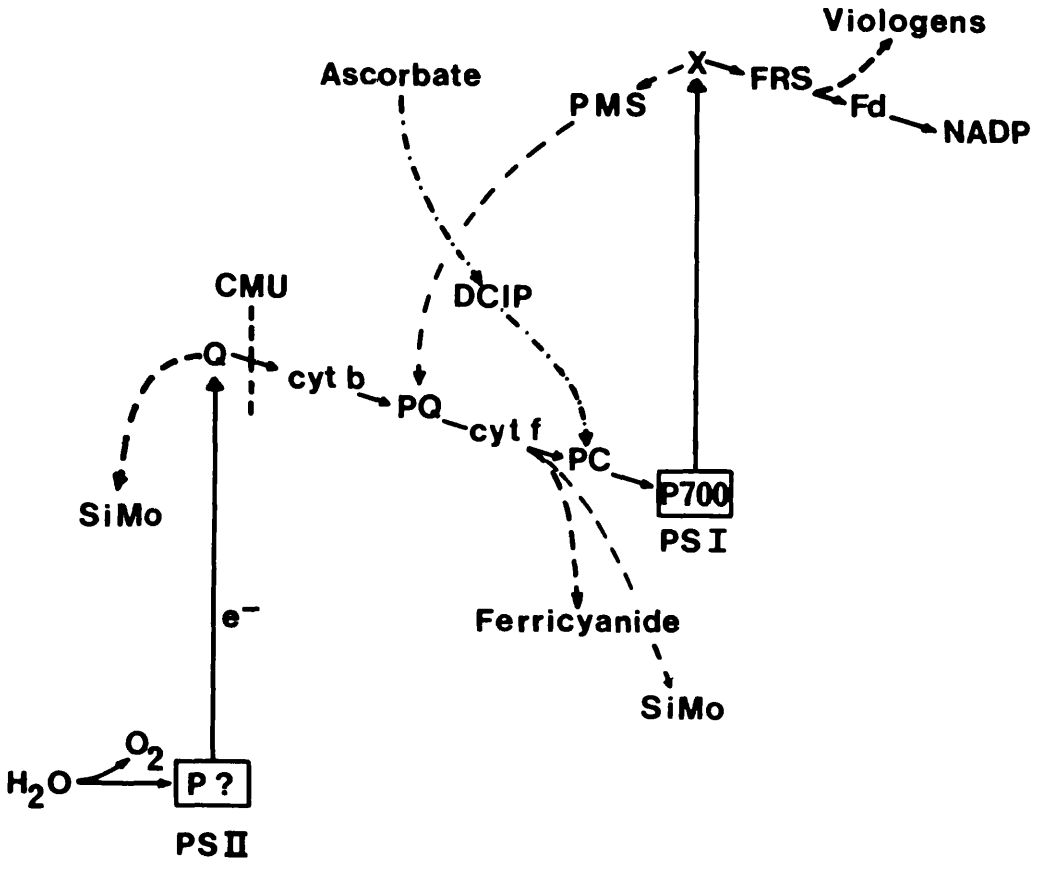


Figure 8. Scheme showing the sites at which artificial electron donors, acceptors and inhibitors are placed in the photosynthetic electron transport chain. SiMo - Silicomolybdic acid; DCIP - Dichloroindophenol; CMU - 3,4 chlorophenyl-1, 1 dimethyl urea; PMS - Phenazine methasulphate.

RESULTS

RESULTS

1. THE EXPLANT AS AN EXPERIMENTAL SYSTEM

The explant is a convenient system to use. The removal of root and cotyledons from the plant, allows rapid uptake of exogenous material which in general is unaffected by endogenous substrates. The pea explant was used, therefore, throughout this experimental work.

Although there are considerable advantages in the use of such a system it must be acknowledged that the plant is no longer under natural conditions and may behave differently. Table 1 shows a selection of results taken from whole leaves and isolated plastids of both intact and excised plants which were both dark grown and greened for 48h under identical conditions. The intact plant synthesised chlorophyll 27% above the level of the explants. This increase in the intact plants must be attributed to substances present in the cotyledons which cannot be substituted by the presence of exogenous sucrose in the experimental explant solution (Hole and Dodge, 1975). The rate of photosynthesis, as measured by CO_2 uptake, was 23.5% higher in intact plants and similarly silicomolybdate reduction, as a measure of PS II activity in isolated chloroplasts, was increased by 27%. These results suggest that the major difference between intact and excised plants is one of chlorophyll levels and it is therefore the higher levels of chlorophyll which are responsible for higher activities in the assays using intact plants. However, PS II activities measured using

TABLE I

	Intact	Excised	% Increase of Intact over Excised plants
Chlorophyll (mg/g.f.wt.)	1.676	1.321	+ 27.0%
Photosynthesis (~~µmols~~ µl CO_2/h/g.f.wt.)	689.0	851.0	+ 23.5%
Ferricyanide PS II (µmols O_2/h/g.f.wt.)	32.10	22.22	+ 44.5%
Silicomolybdate PS II (µmols O_2/h/g.f.wt.)	30.87	24.369	+ 27.0%
PS I (Asc. photooxidation/µmols O_2/h/g.f.wt.)	77.195	96.250	- 24.7%

Electron transport reactions from intact and excised pea plants after 48h continuous illumination.

ferricyanide as a terminal electron acceptor are somewhat higher and cannot be readily explained.

In contrast to the stimulated rate of activity in the intact plants, the rate of PS I activity as measured by ascorbate photo-oxidation was reduced by almost 25%. This can be explained by excitation energy spillover from PS II to PS I (see Discussion section 4).

2. GROWTH AND EXPERIMENTAL CONDITIONS

Although the explant system does not behave as naturally as might be desired, it is advantageous not only for reasons mentioned in results section 1 but also in that experimental variation can be reduced to a minimum by optimisation of the growth and experimental parameters. The determination of the latter was based on four experimental systems under which a comparative functional and structural study of chloroplast development was to be carried out. These were as follows:

a) The presence of photosynthesis (water control system).

b) The absence of photosynthesis (CMU).

c) The presence of photosynthesis and an exogenous substrate (water/sucrose).

d) The absence of photosynthesis but with the presence of an exogenous substrate (CMU/sucrose).

Standardisation of the parameters in this section was carried out using final chlorophyll levels in the explants as a simple

qualitative criterion for assessing chloroplast development.

2.1 Removal of exogenous substrate

The synthesis of chlorophyll and associated chloroplast development is dependent upon a supply of substrates (Wolfe and Price, 1957). Under normal circumstances the substrate in a pea seedling is initially translocated from the cotyledons to the primary leaves in the form of carbohydrates. Resultant photosynthetic competence eventually substitutes for the cotyledon substrate and further chloroplast development will follow. In the present study where the role of photosynthesis in the development of the chloroplast was the major concern, there was no requirement for competition from endogenous substrate. Although the primary source was removed during excision substantial amounts would still have been present in the shoot. This substrate could be removed by dark incubation in the experimental solutions, a procedure which also effected their uptake prior to illumination. The inhibitory effect of CMU on chlorophyll synthesis was shown to be reversed by the presence of carbohydrate (Klein and Neuman, 1966) and it was therefore possible to utilise this system to monitor the removal of endogenous substrate. Hence when all the latter had been removed chlorophyll synthesis would be totally inhibited by CMU.

The removal of endogenous substrate from etiolated pea explants is shown in figure 9. Since there was a direct relationship between the amount of substrate present and the length of dark

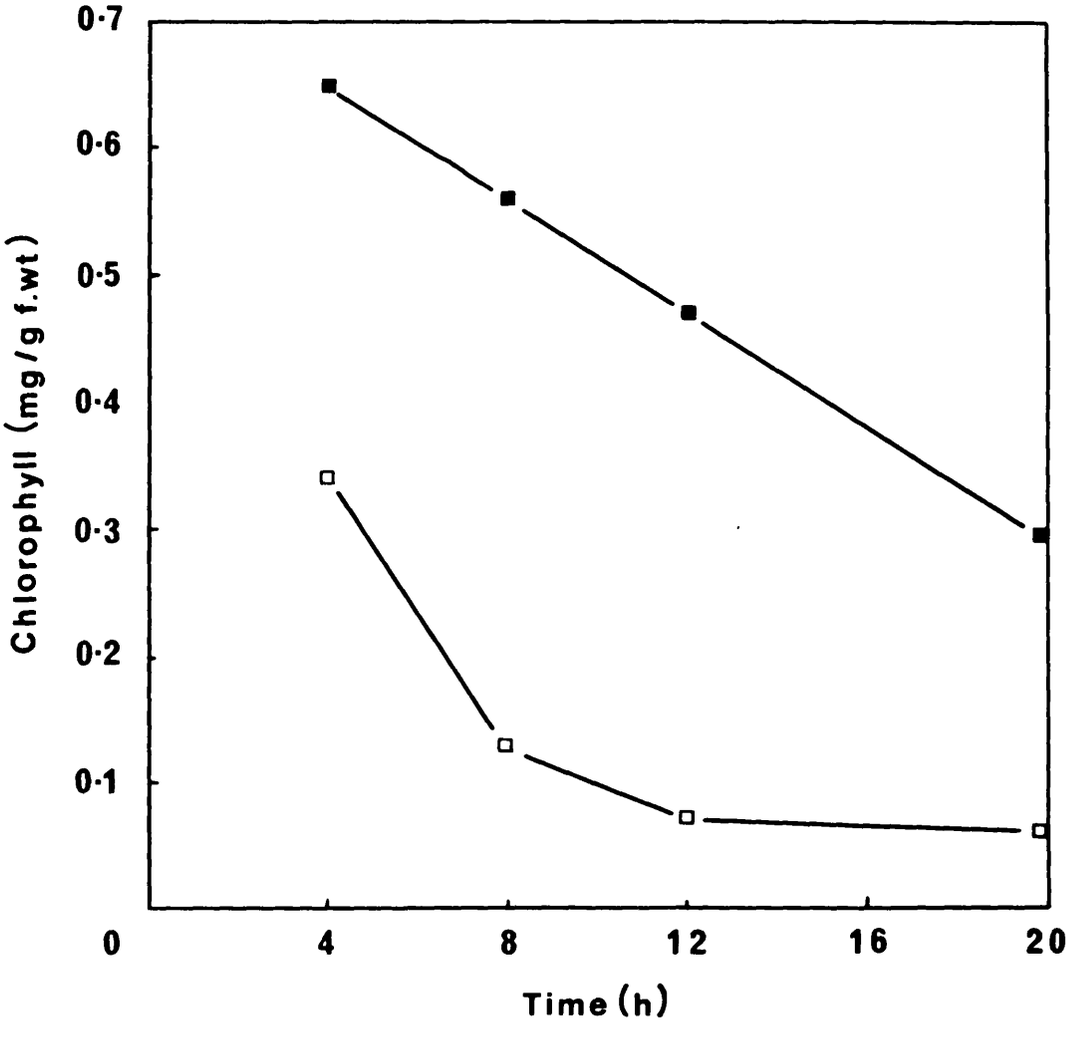


Figure 9. The effects of shoot length and increasing dark pre-incubation time on the removal of endogenous substrate in excised pea plants. (□) 5 cm; (■) 12 cm.

incubation time, both were reduced by shortening the length of the explant shoot. Explants were cut with shoot lengths of twelve and five cm and preincubated in darkness for increasing times. Chlorophyll levels of the treatments were determined after 48h continuous illumination.

Figure 9 shows that the chlorophyll levels of explants of both shoot lengths decreased as the preincubation time was increased. Chlorophyll levels of the longer shoot length treatment decreased linearly but substantial amounts were synthesised after 20h preincubation. By contrast explants of the shorter shoot length synthesised similar chlorophyll levels after only 4h dark preincubation and almost total inhibition of chlorophyll synthesis was effected after 12h. In subsequent experimental work pea plants were excised five cm below the apical hook and for convenience were preincubated in darkness overnight (16h).

It should be pointed out that increased inhibition of chlorophyll synthesis with preincubation time may be due instead to translocation of CMU to its site of action or a combination of this and the removal of substrates. However, results are available which show that CMU uptake by freshly excised plants could occur within several minutes and would certainly be complete within 4h (Pallett-unpublished data).

2.2 Optimum treatment concentrations

Optimum concentrations of CMU and sucrose were determined by their ability to inhibit and reverse CMU inhibited chlorophyll synthesis respectively. Figure 10 shows the effect of increasing CMU concentration on the chlorophyll content of pea explants after 48h continuous illumination. Etiolated pea plants were excised five cm below the apical hook and preincubated in darkness for 16h in CMU solutions of increasing concentration. An approximately linear decrease in leaf chlorophyll was obtained as CMU concentrations were increased and a maximum CMU effect occurred at a concentration of 5×10^{-4}M. A further increase in CMU concentration was ineffective in producing additional inhibition of chlorophyll synthesis. The small level of chlorophyll present at high CMU concentrations was probably synthesised from the photoconversion of protochlorophyllide present in the prolamellar body.

Figure 11 shows the reversal of CMU inhibited chlorophyll synthesis by the addition of exogenous sucrose. Etiolated pea plants were preincubated in darkness for 16h in solutions containing CMU (5×10^{-4}M) and with increasing sucrose concentrations. Chlorophyll levels were determined after 48h continuous illumination. CMU inhibition of chlorophyll synthesis was unaffected by a sucrose concentration of 5×10^{-6}M. Reversal however was observed at all concentrations of sucrose thereafter with maximum chlorophyll synthesis occurring at 5×10^{-2}M sucrose. A further increase in sucrose concentration above 5×10^{-2}M produced a sharp decrease in leaf

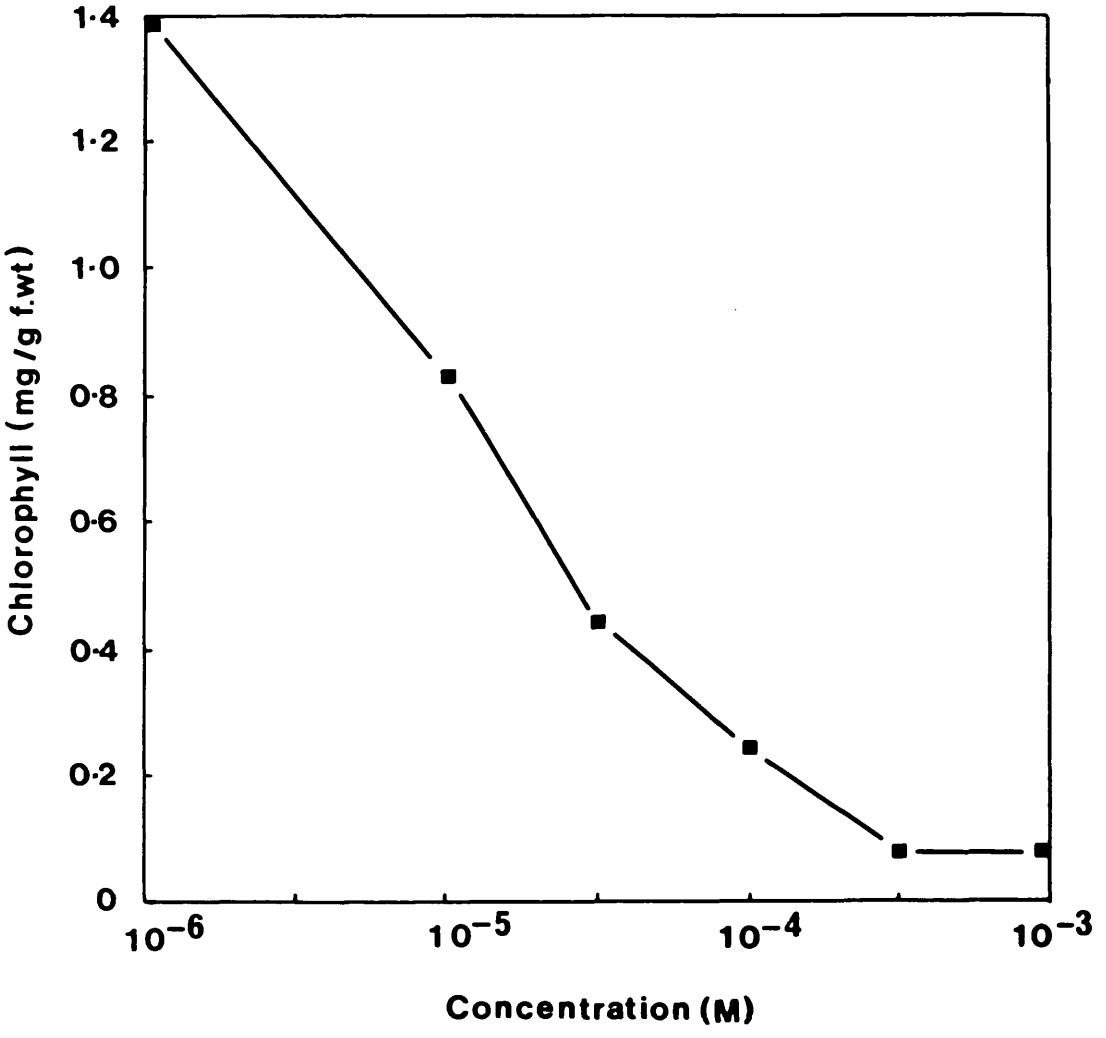


Figure 10. The determination of the optimum CMU concentration required for maximum inhibition of chlorophyll synthesis.

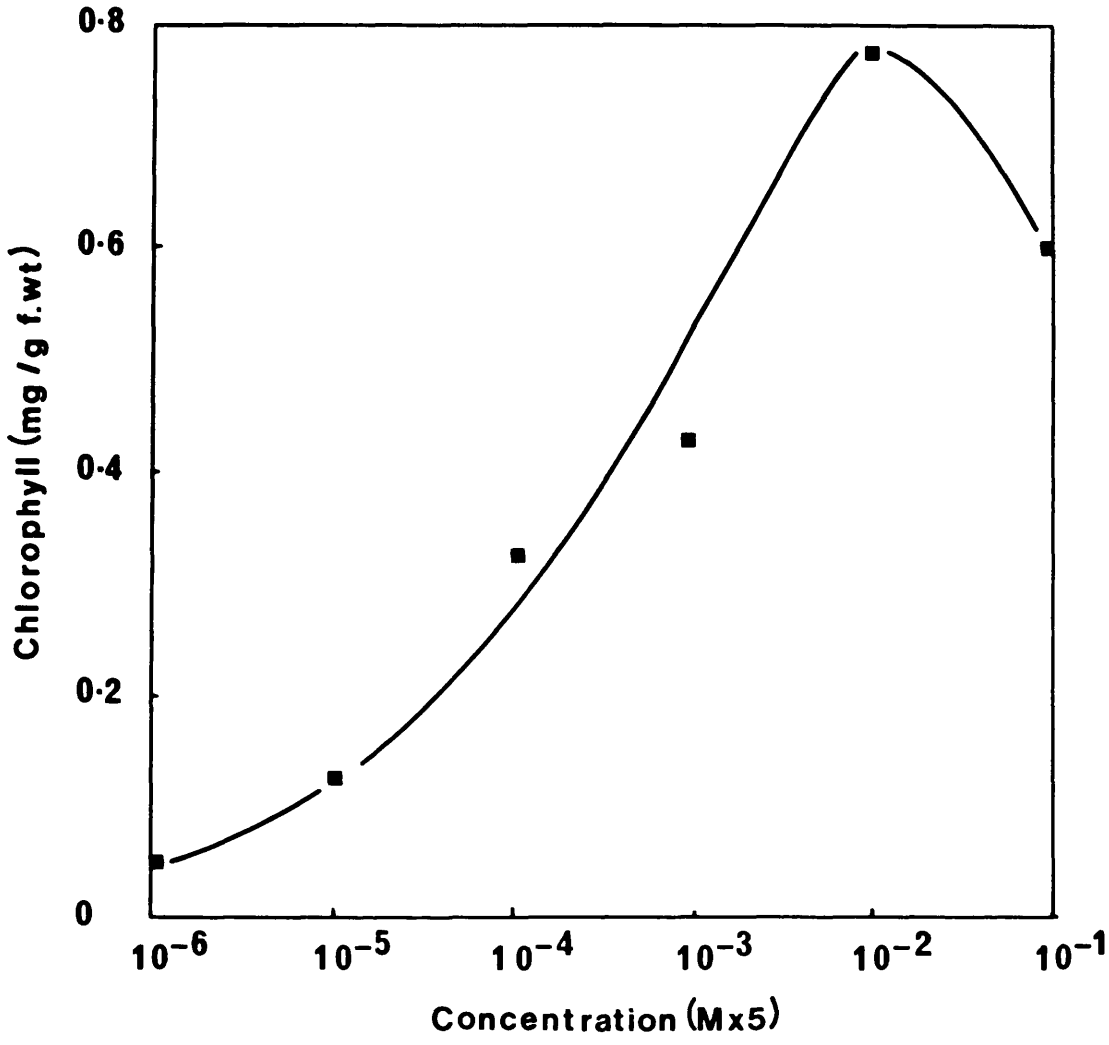


Figure 11. The determination of the optimum sucrose concentrations to produce maximum reversal of CMU inhibited chlorophyll synthesis.

chlorophyll content. This was thought to be due to cellular osmotic effects by high concentrations.

CMU and sucrose concentrations used in the subsequent experiments were maintained at 5×10^{-4}M and 5×10^{-2}M respectively.

2.3 Age of dark grown material

The age at which the etiolated pea plants were harvested was shown to affect subsequent chlorophyll synthesis (figure 12). Plants excised after 10 days growth at 22.5°C and preincubated with sucrose (5×10^{-2}M) for 16h, synthesised chlorophyll to a level approximately 40% of those excised after 7 days when greened for 48h. The inability of longer dark grown plants to maintain chlorophyll synthetic rates equivalent to the plants grown for shorter periods would suggest senescence during dark growth.

Plants chosen for subsequent experimental use therefore, were those which produced as near maximal chlorophyll contents as possible and which were of sufficient size to permit the growth of manageable quantities of starting material. Etiolated pea plants grown for 8 days at 22.5°C produced substantially larger primary leaves than those at 7 days, although the time course for chlorophyll synthesis was similar. Dark grown pea plants of this stage were therefore used in all subsequent experiments.

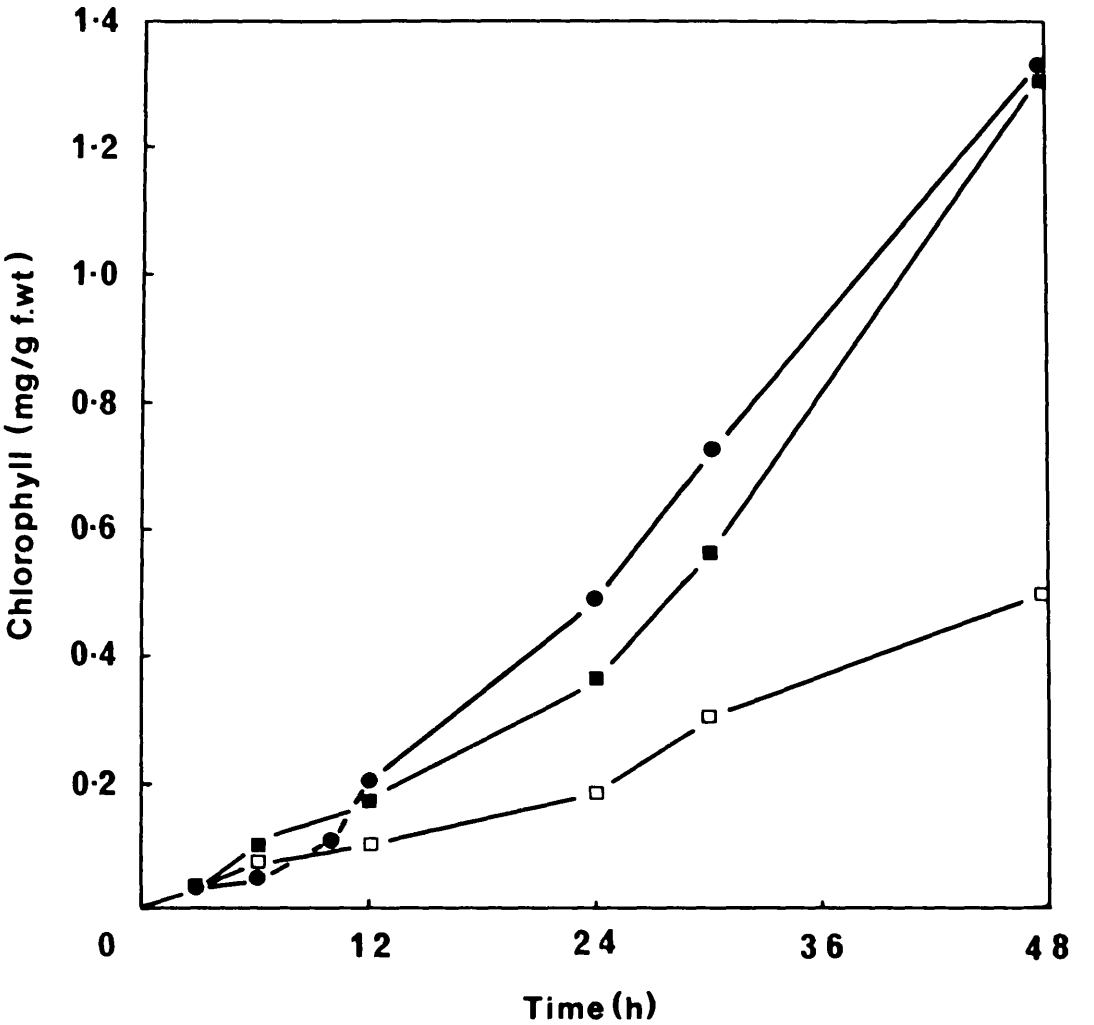


Figure 12. The effect of age of dark grown plants on the subsequent ability of pea explants to synthesise chlorophyll. Plants were dark grown for (●) 7 days; (■) 8 days; and (□) 10 days prior to excision and dark preincubation in sucrose (5 x 10^{-2}M) for 16h.

2.4 Optimisation of light intensity

The effects of increasing light intensity on the chlorophyll synthetic rates of the four comparative treatments are shown in figure 13. Etiolated plants were excised after 8 days growth at 22.5°C and preincubated for 16h in water or solutions containing CMU (5×10^{-4}M) and sucrose (5×10^{-2}M) as required. White light of 24 W/m^2 was provided by warm white fluorescent tubes and the intensity varied using neutral density filters (Kodak Ltd.).

Figure 13 shows that the light intensity maxima for chlorophyll synthesis varied greatly according to the plant treatment. In general it may be considered that the maxima are a result of two opposing light mediated reactions. The increase in chlorophyll content with light was a result of photoconversion of substrates to chlorophyll. In contrast the decrease seen at higher light intensities was probably due to photooxidation of chlorophyll. The optimum light intensity for each treatment therefore was a balance of these two factors.

Absence of photosynthesis in the CMU treatment resulted in the production of small chlorophyll contents at a low light intensity of approximately 1 W/m^2. This chlorophyll was synthesised from the protochlorophyll(ide) present in the prolamellar body of the etioplasts, substrate for further chlorophyll synthesis not being available. 1 W/m^2 may therefore be considered as the threshold light intensity for the conversion of substrates to chlorophyll in pea explants. Light intensities above 1 W/m^2 were seen to cause chlorophyll photodestruction in this treatment but with the other treatments chlorophyll photo-

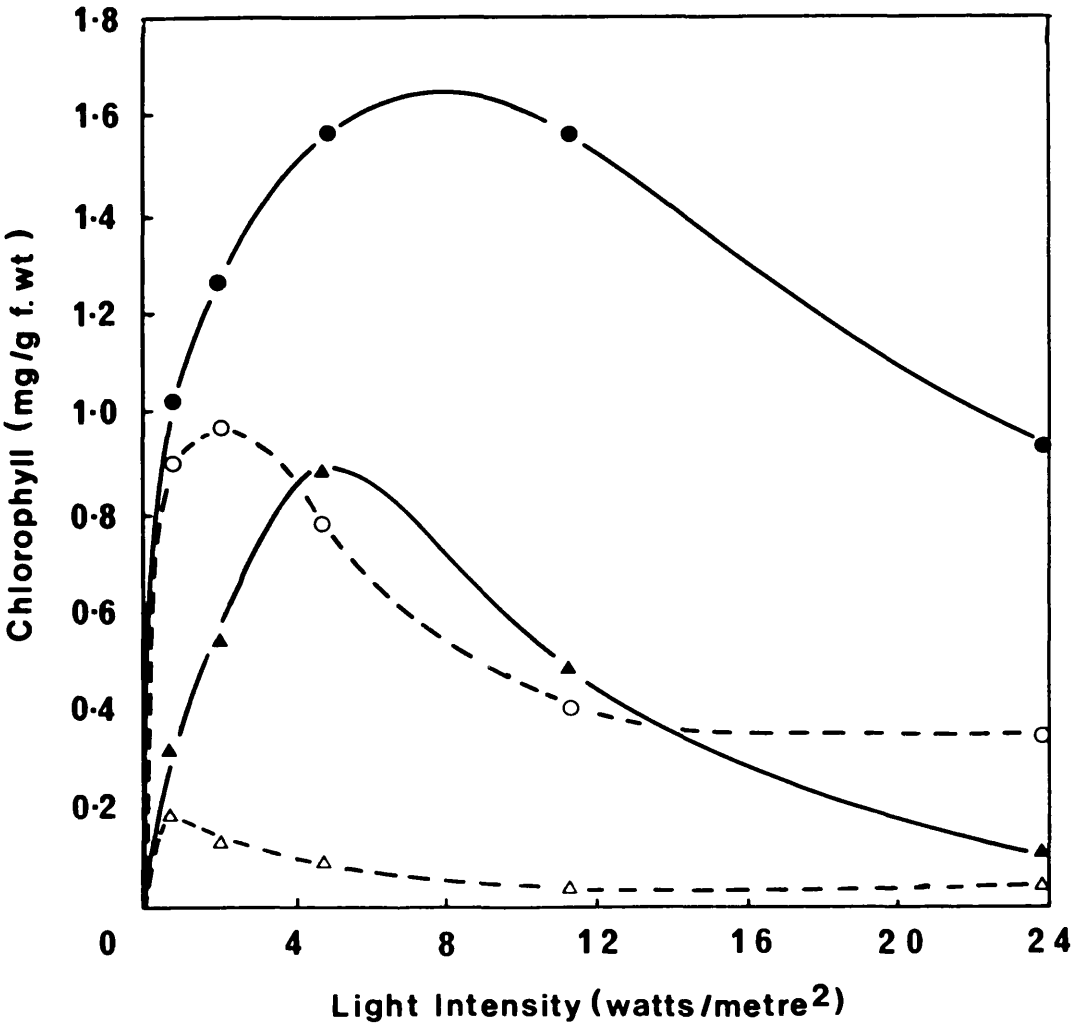


Figure 13. Graph showing the effects of increasing light intensity on the synthesis of chlorophyll in treated etiolated pea explants. (●) water/sucrose; (○) CMU/sucrose; (▲) water control; (△) CMU.

destruction could have been masked by higher rates of synthesis.

Addition of CMU and sucrose produced a large increase in chlorophyll synthesis at threshold light intensity. The maximum , however, was broader and photodestruction of chlorophyll was not observed until after 3 W/m^2.

The presence of photosynthesis, without an additional supply of endogenous substrate, was shown by the water control. Chlorophyll synthesis in this treatment was due solely to a supply of substrates by photosynthesis, the rate of which was dependent upon light intensity. The light intensity maximum for chlorophyll synthesis in this treatment may also be regarded as the light intensity which produces the highest photosynthetic rates.

The effects of combining exogenous and photosynthetic substrates is shown by the light intensity maximum of the water/sucrose treatment. The peak was broad and appeared not only to combine the maxima of the water control and CMU /sucrose treatments to produce a maximum between 3 and 5 W/m^2 but also combined the chlorophyll contents of both treatments. The large increase in chlorophyll content of the water/sucrose treatment showed a light intensity maximum of 12 W/m^2 before chlorophyll photodestruction was detected.

As a result of the large variation in light intensity maxima between the four treatments, an optimum light intensity of 4 W/m^2 was used for plant illumination in all subsequent experiments.

3. PIGMENT CHANGES

3.1 Chlorophyll time course

Under the conditions established in the previous section the time courses for chlorophyll synthesis in treated explants are shown in figure 14. Low levels of chlorophyll were detected in all treatments after 3h illumination and may be attributed to the photoconversion of protochlorophyllide present in the etioplast. Provided that all endogenous substrate was removed during preincubation of the treatments little other chlorophyll synthesis could be detected in the CMU treated plants over the 48h illumination period. A lag period during which little chlorophyll was synthesised was observed between 3 - 6h in the sucrose-containing treatments but was extended to 9h in the water control. These results are consistent with those of Sisler and Klein(1963). Addition of sucrose to the CMU treatment reversed the CMU inhibition to produce chlorophyll levels approximately double those of the water control. A greater enhancement of chlorophyll levels was obtained by similar addition of sucrose to the water control. After 48h the chlorophyll contents of this treatment were approximately 150% greater than the water control and 50% above the CMU/sucrose treatment. Similar differences were seen at each point in the timecourse after 12h illumination. From 3h to 12h illumination however the differences between the CMU/sucrose and H_2O/sucrose treatments were negligible, and it may be concluded that photosynthesis in the latter was not efficient enough at this point to enhance the chlorophyll content

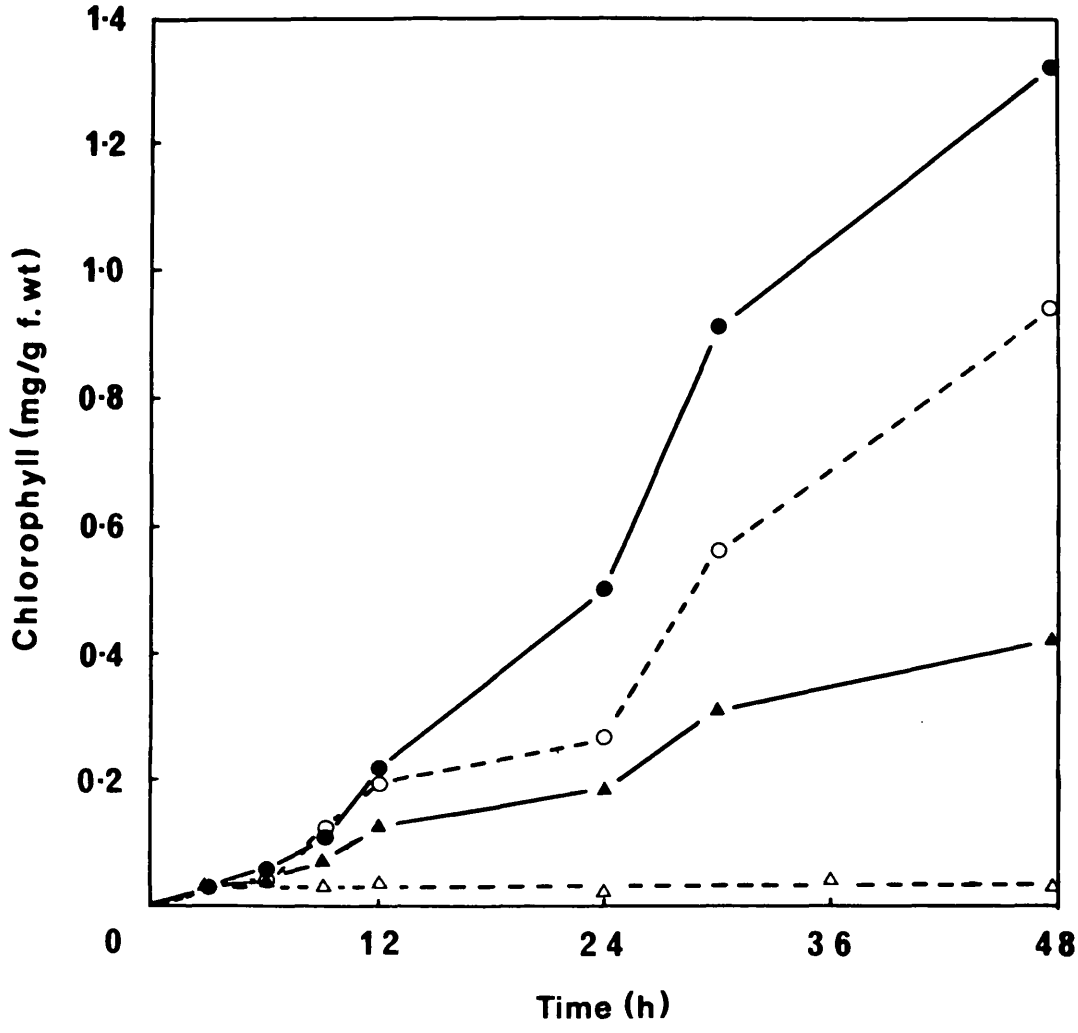


Figure 14. Time course showing the changes occurring in the total chlorophyll content of etiolated pea explants over a period of 48h continuous illumination. Chlorophyll levels were determined as described in Methods 5.3.1. (●) water/sucrose; (O) CMU/ sucrose; (▲) water control; (△) CMU.

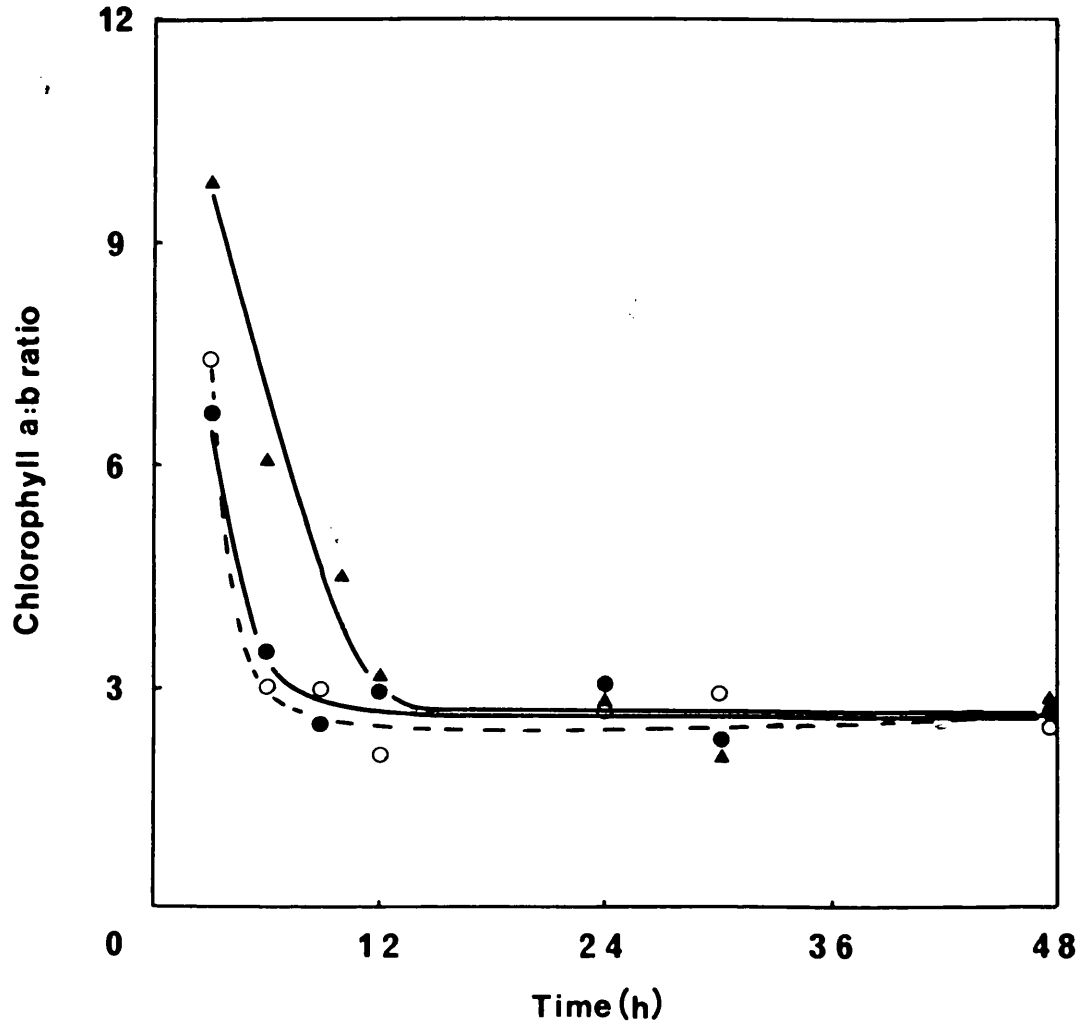


Figure 15. Changes occurring in the chl a:b ratio in etiolated pea explants over a period of 48h continuous illumination. Chlorophylls a and b were determined as described in Methods 5.3.1. (●) water/sucrose; (○) CMU/sucrose; (▲) water control; (△) CMU.

above that of the sucrose-containing CMU treatment.

The ratio of chlorophyll a to chlorophyll b was shown to change during the 48h illumination period (Figure 15). After 3h the chlorophyll a:b ratio of both CMU/sucrose and H_2O/sucrose treatments was approximately 7:1. The ratio decreased with time, reaching a minimum of approximately 3:1 between 6h and 9h. This ratio remained constant during subsequent illumination. The water control followed a similar pattern, having a higher chlorophyll a:b ratio of 10:1 at 3h, which decreased with further illumination. The ratio of 3:1 was not reached in this treatment until approximately 12h illumination. The difference of time in assuming this minimum chlorophyll a:b ratio of 3 between the sucrose-containing treatments and the water control, may be explained by a difference in the rate of pigment development. There was also shown to be a difference in lag period in the chlorophyll time course (Figure 14).

3.2 Carotenoid changes

The carotenoid pigments were separated by thin-layer chromatography (see Methods section 5.4) into a single band containing both α and β carotene and 4 - 6 xanthophyll bands of which the major band contained an abundance of lutein. The extracted carotenoids were quantified using the extinction coefficient of the predominant pigment of the mixture since these measurements reflected the relative changes in the total carotenes and xanthophylls. Hence, the absorption spectra of the carotene band showed a predominance of β-carotene and the major

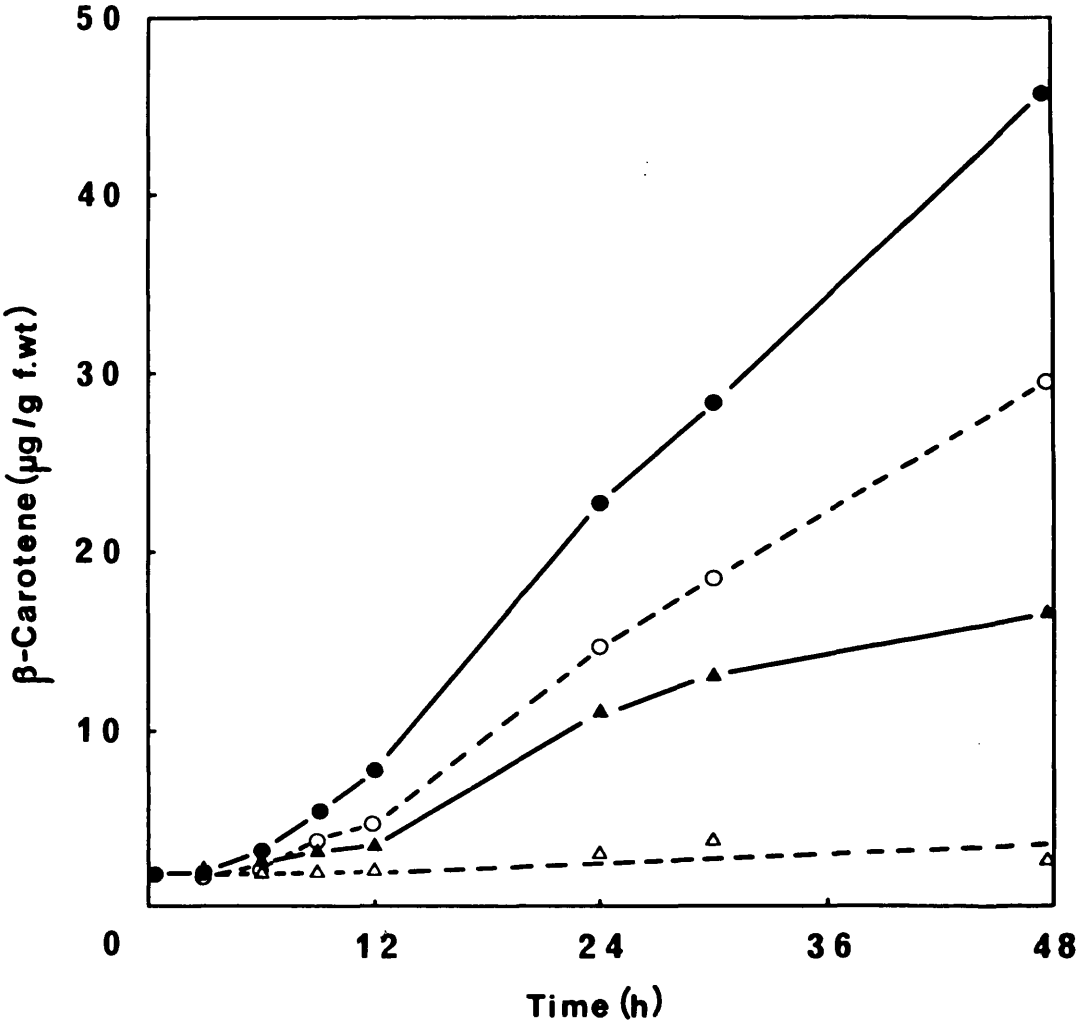


Figure 16. Time course showing the changes occurring in the β-carotene content of etiolated pea explants during a period of 48h continuous illumination. The pigment content was resolved and determined as described in Methods 5.4. (●) water/sucrose; (○) CMU/sucrose; (▲) water control; (△) CMU.

xanthophyll band contained an abundance of lutein.

Figure 16 shows the changes in β-carotene levels in treated explants during 48h continuous illumination. Low but significant dark levels were measured in all etiolated treatments. The CMU treatment produced little change in β-carotene with the onset of illumination whereas the other treatments, after an approximate 3h lag period, increased steadily over the 48h. The inhibitory effect of CMU on β-carotene was reversed by the addition of sucrose to levels 67% above the water control after 48h. Similar addition of sucrose to the water control however increased the β-carotene level by 150%.

Changes in the lutein contents of the treatments over a period of 48h illumination showed a somewhat different trend (Figure 17). Unlike β-carotene dark levels were found to be considerably higher at 20 µg/g. fresh weight. In addition the lag period in lutein synthesis after the onset of illumination was comparable with that of chlorophyll (Figure 14) and lasted for approximately 6 - 9h. Active lutein synthesis occurred after 12h in all treatments other than the CMU treated explants, reaching a maximum at 30h. Between 30 and 48h a parallel decrease in lutein content was obtained in all treatments. Once again, as with chlorophyll (Figure 14), and β-carotene (Figure 16) little change was observed in the overall lutein content of the CMU treatment. After a small increase over the first 12h illumination a slow but steady decrease occurred. Similarly the high levels of lutein synthesised in the H_2O/sucrose treatment are approximately equivalent to the cumulation of contents synthesised in the CMU/sucrose and water treatments.

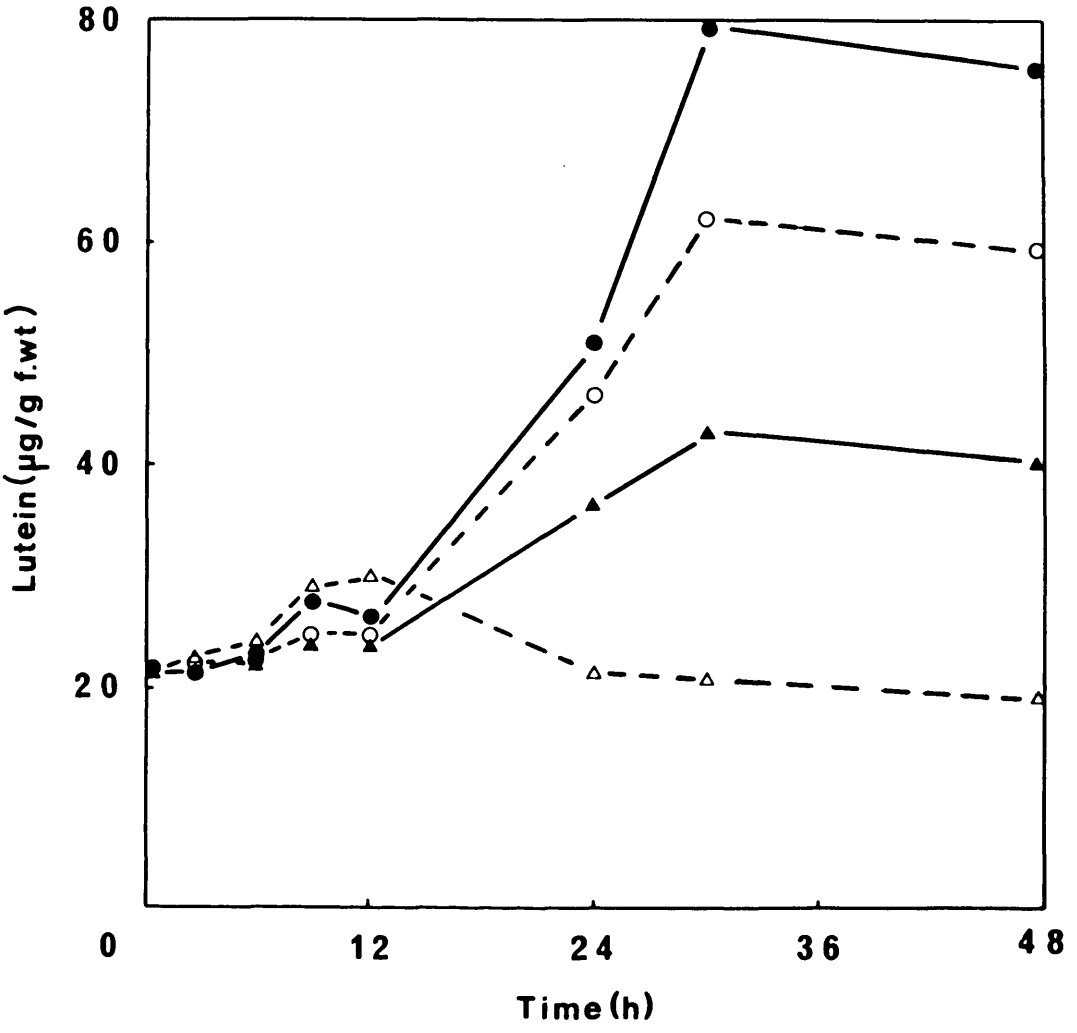


Figure 17. Time course showing the changes occurring in the lutein content of etiolated pea explants during 48h continuous illumination. The pigment was resolved and determined as described in Methods 5.4. (●) water/sucrose; (○) CMU/sucrose; (▲) water control; (△) CMU.

4. DEVELOPMENT OF FUNCTIONAL ACTIVITY

4.1 Whole plant photosynthesis

The effect of CMU as an inhibitor of whole plant photosynthesis is shown in figure 18. Plant material was grown and treated as described in the Results section 1. Photosynthesis was measured by determining CO_2 uptake using the infra red gas analysis system. CO_2 uptake was initiated in both water control and water/sucrose treatments approximately 5h after the onset of illumination. The rates at which photosynthetic competence subsequently developed differed greatly between these two treatments. In the water treatment a lag occurred after the initial onset of photosynthesis and the rate remained unchanged until 12h illumination. Similar results were shown by Wolfe and Price (1957) also using explants. The presence of sucrose however abolished the lag phase and after 12h illumination the rate was four-fold higher than that of the water treatment. Maximum photosynthetic rates also varied between these two treatments, with the H_2O/sucrose reaching a maximum after 30h and the H_2O remaining on an upward trend at 48h.

CMU and CMU/sucrose treatments remained totally inhibited during this illumination period. Since CMU treated plants were incapable of accumulating chlorophyll (figure 14) it was to be expected that photosynthesis could not develop. However, addition of sucrose, although capable of reversing chlorophyll inhibition, did not have a similar role in the inhibition of photosynthesis.

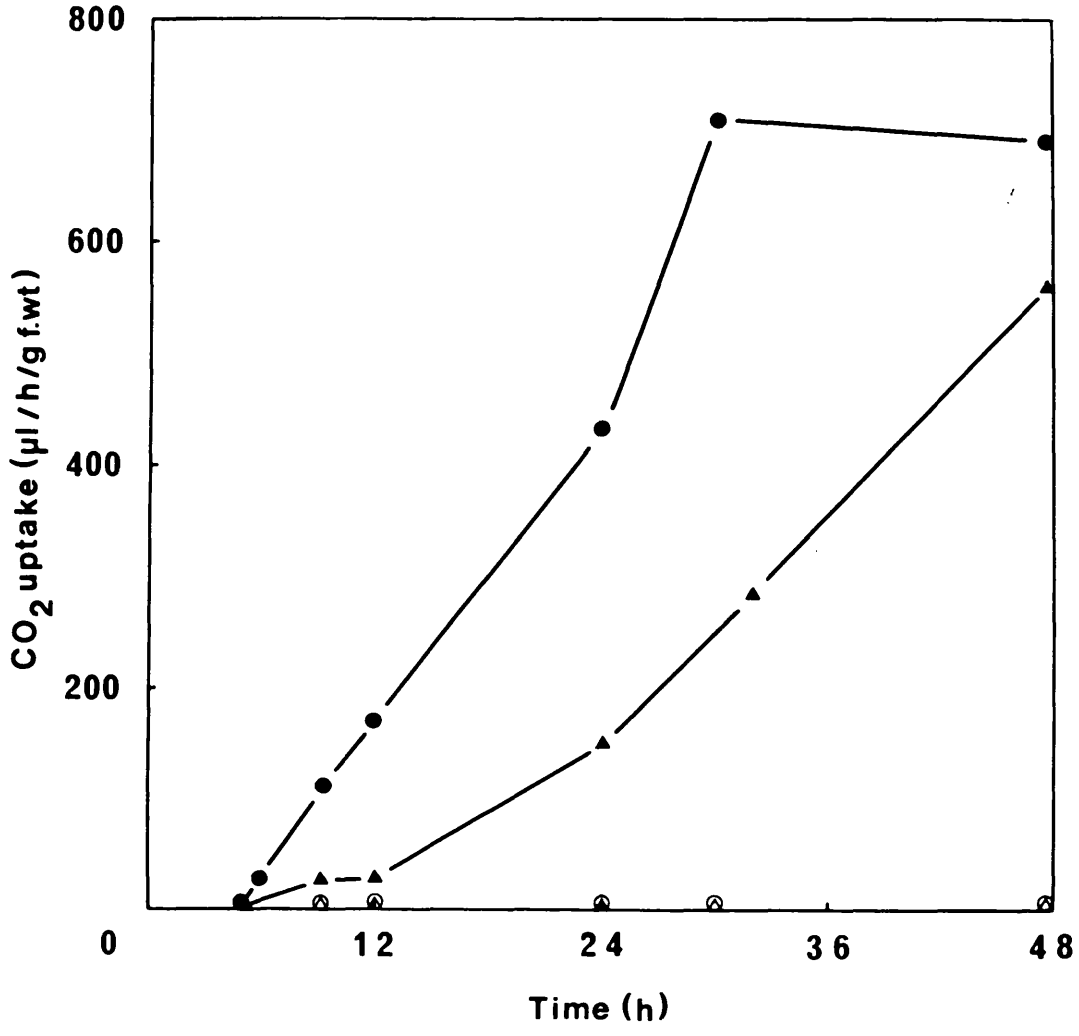


Figure 18. Time course showing the onset and development of CO_2 uptake in etiolated pea explants over a period of 48h. CO_2 uptake was measured in the IRGA (see Methods 4). (●) water/sucrose; (○) CMU/sucrose; (▲) water control; (△) CMU.

4.2 Partial electron transport reactions

4.2.1 PS I and PSII development

Figure 19 shows the whole chain (PS I and PS II) electron transport activities in chloroplasts isolated from photosynthetically competent and incompetent plants. Chloroplasts were isolated after the required illumination period and assayed using water as the natural electron donor and methyl viologen (paraquat) as the terminal acceptor (see figure 8). The overall reaction was determined by a net oxygen uptake in an oxygen electrode.

Activities were detected only in the water control and water/sucrose treatments. Both treatments containing CMU were totally inhibited during the period of illumination. These results are therefore consistent with the theory that the primary role of CMU as a photosynthetic inhibitor is by blocking electron flow between the primary electron donor to PS II and the more redox negative electron carriers of PS I.

Initiation of electron flow in the water and water/sucrose treatments occurred at approximately 5h and may be compared to the onset of CO_2 uptake (figure 18). Once again there was evidence of a greater lag period in the treatment without sucrose and overall lower activities generally occurred. Maximum activities were seen in both treatments at 30h after which time a decline in the rates possibly reflected severe ageing of the explants.

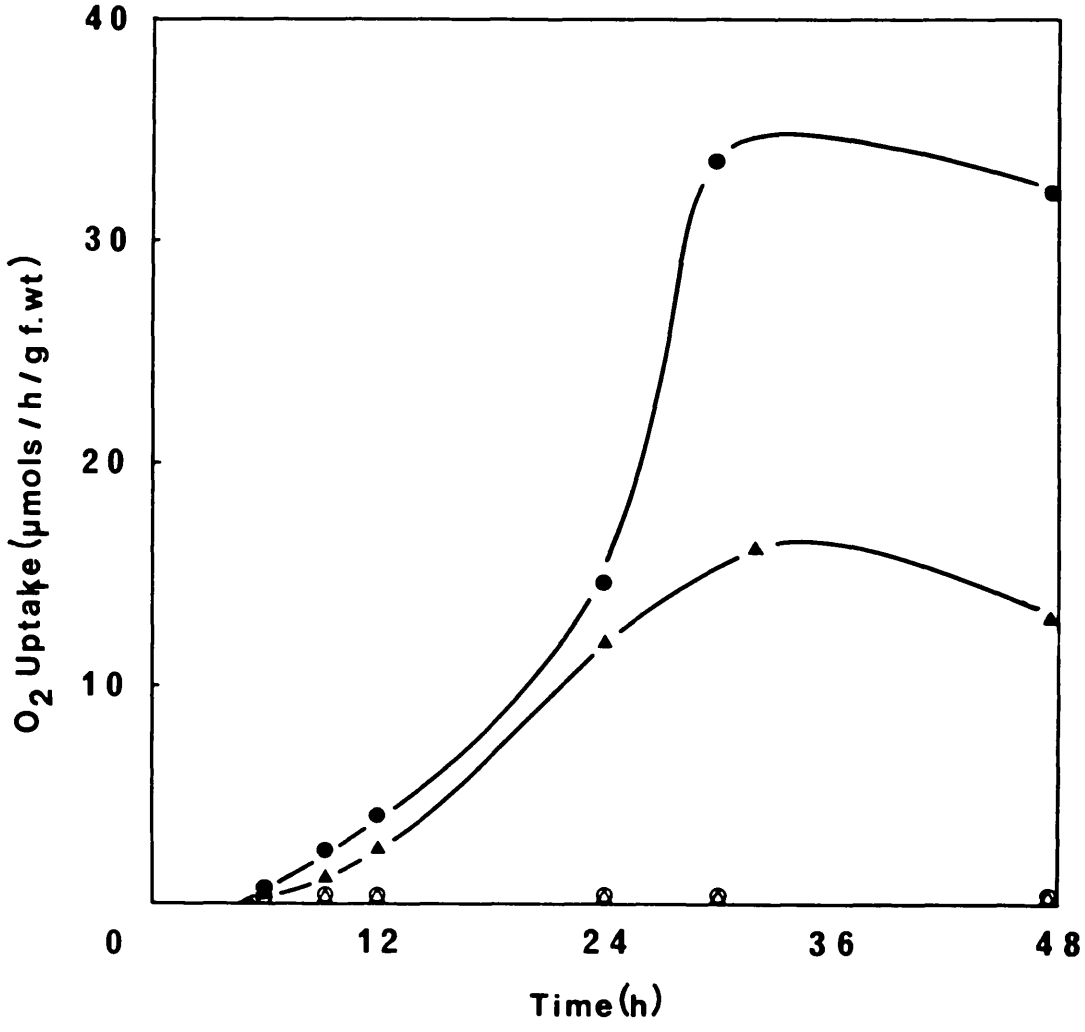


Figure 19. Time course showing the development of whole chain electron transport (PS II and PS I) in plastids isolated from greening pea explants over a period of 48h continuous illumination. Paraquat was used as the terminal electron acceptor from water and net O_2 uptake was measured in an O_2 electrode. (●) water/sucrose; (○) CMU/ sucrose; (▲) water control; (△) CMU.

4.2.2 PS II activity

The measurement of PS II development over a period of 48h produced similar results. Chloroplasts isolated from plants treated as previously described, were assayed for PS II activity using ferricyanide as a terminal electron acceptor. Photosynthetic oxygen evolution was measured in an oxygen electrode over a period of 48h illumination (figure 20).

PS II activities were initiated after approximately 5h illumination and these results would suggest that PS II is the limiting factor in the onset of CO_2 uptake (figure 18) and whole chain electron transport (figure 19). The time courses showed that electron transport was detected only in the treatments which lacked CMU. This confirmed the view that this photosynthetic inhibitor has a site of action in the vicinity of PS II. Acceleration in the rates of activity occurred after 12h and maxima were once again reached after 30h. The development of PS II activity, however, seemed little affected by the presence of sucrose since both the H_2O and H_2O/sucrose rates were remarkably similar.

Measurement of PS II activity using SiMo as an electron acceptor has provided additional information on the development of functional electron transport in CMU treated plants. Silicomolybdate has been shown to accept electrons from Q at a point prior to the site of action of CMU. Figure 21 shows the time course for the development of PS II using this assay system (Barr et al., 1975). The familiar sigmoidal development of PS II was once again demonstrated for both

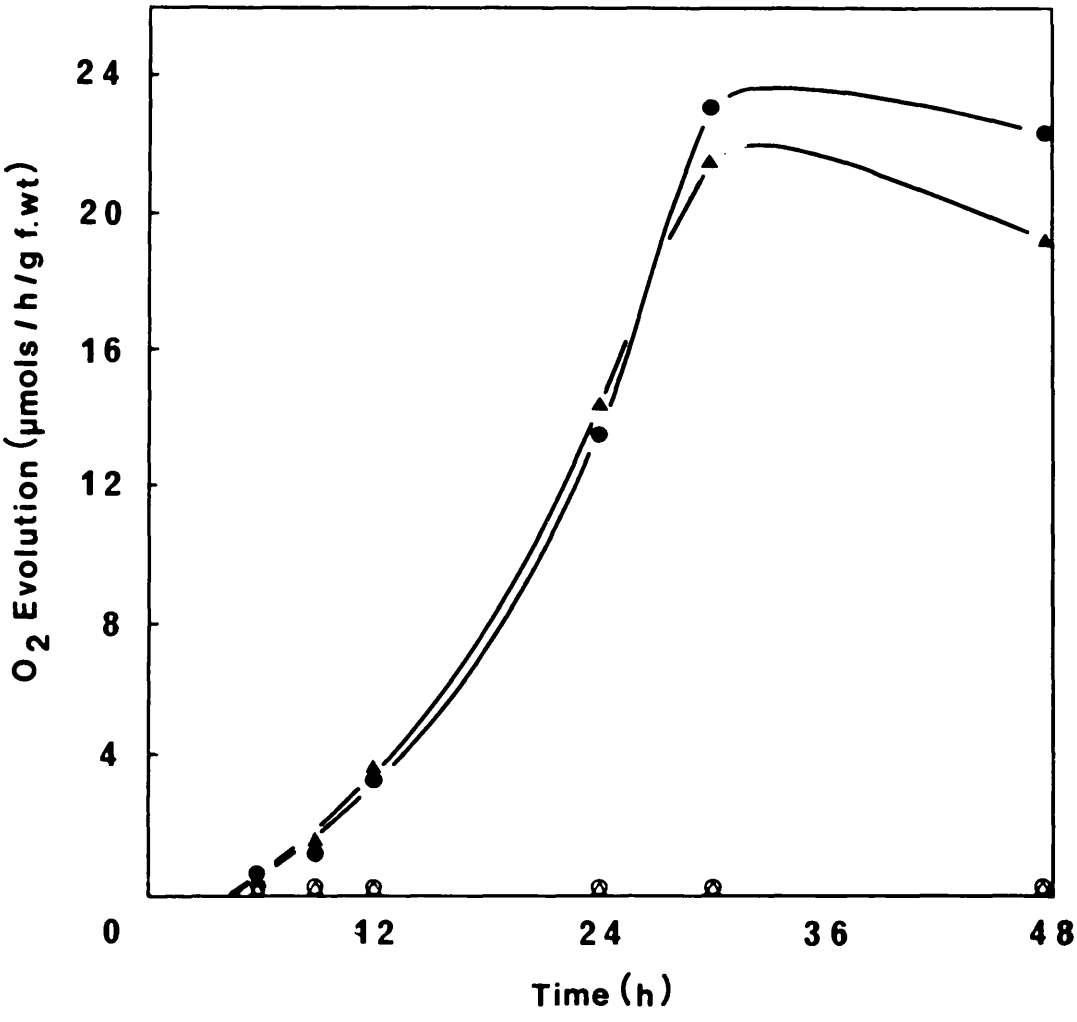


Figure 20. Time course for the onset and development of PS II activity as measured by ferricyanide reduction, in plastids isolated from greening pea explants over a period of 48h continuous illumination. O_2 evolution was measured in an O_2 electrode. (●) water/sucrose; (○) CMU/sucrose; (▲) water control; (△) CMU.

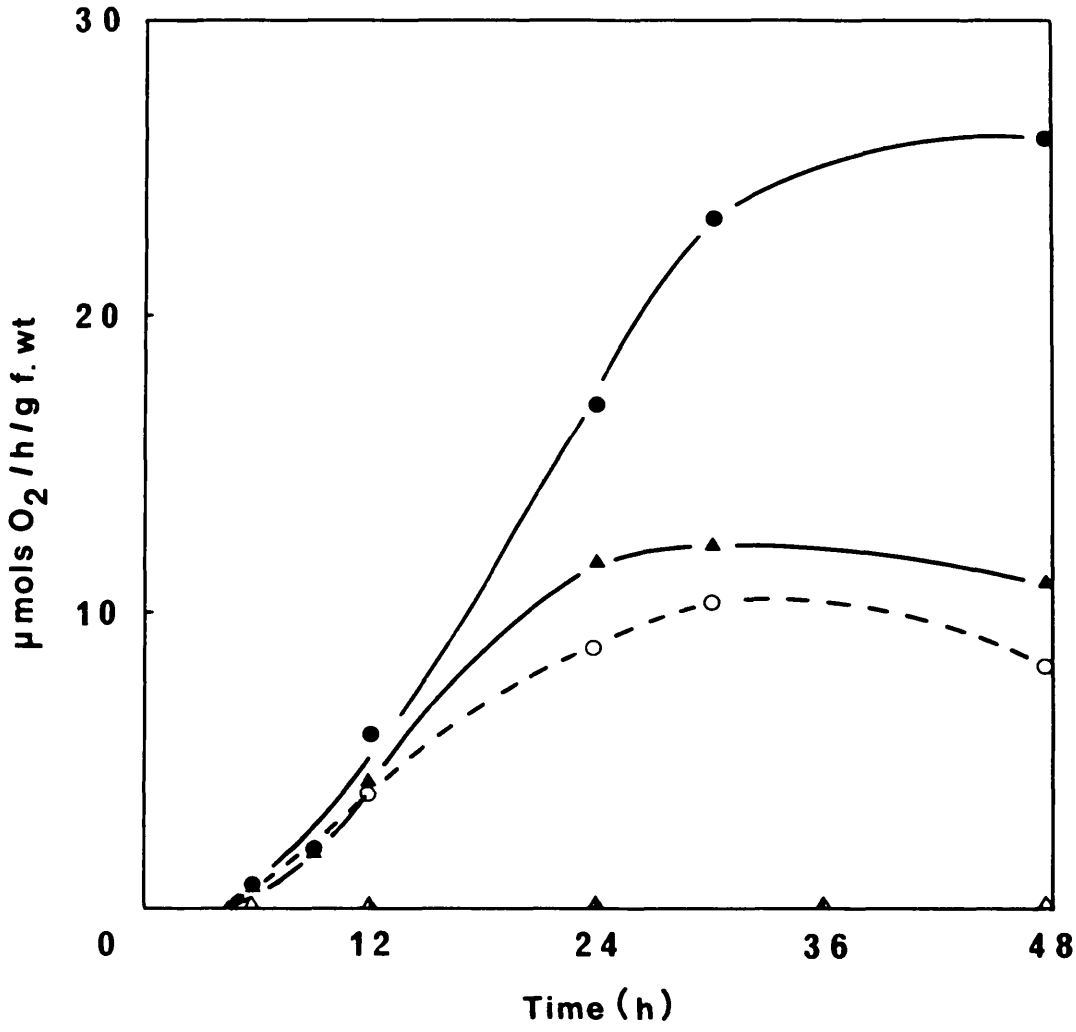


Figure 21. Time course for the onset and development of PS II activity as measured by SiMo reduction, in plastids isolated from greening pea explants during 48h continuous illumination. O_2 evolution was measured in an O_2 electrode. (●) water/sucrose; (O) CMU/sucrose; (▲) water control; (△) CMU.

the H_2O and H_2O/sucrose treatments over the 48h illumination period. In addition, however, the onset and development of PS II activity was observed in the chloroplasts isolated from plants treated with CMU and sucrose. Nevertheless the rates were somewhat lower than the H_2O control and considerably lower than the H_2O/sucrose treatment which contained comparable chlorophyll contents. These deceptively low rates may be explained by silicomolybdate having two sites of electron acceptance before and after Q (Giaquinta and Dilley, 1975). The H_2O and H_2O/sucrose rates utilise both silicomolybdate sites and are therefore maximal. By contrast the CMU/sucrose treatment would only utilise the site prior to Q and hence the rates were approximately half maximal.

The development of the oxygen evolving centres of PS II was measured by the ability of the treatments to incorporate manganese into the lamellae during greening. Figure 22 shows manganese incorporation into etioplasts during the 48h illumination period. The results show that a considerable amount of manganese is present in the prolamellar body of the etioplast. It is concluded that this manganese is responsible for membrane structuration and hence subsequent increases in manganese content during illumination were concomitant with the formation of PS II oxygen evolving centres. The lower initial manganese contents of the CMU/sucrose treated lamellae is difficult to explain.

Manganese incorporation was found to be greatest in those treatments containing sucrose and therefore the presence of CMU in the CMU/sucrose treatment produced no inhibitory effect. Lower manganese uptake was demonstrated in the control and the CMU treatment showed a

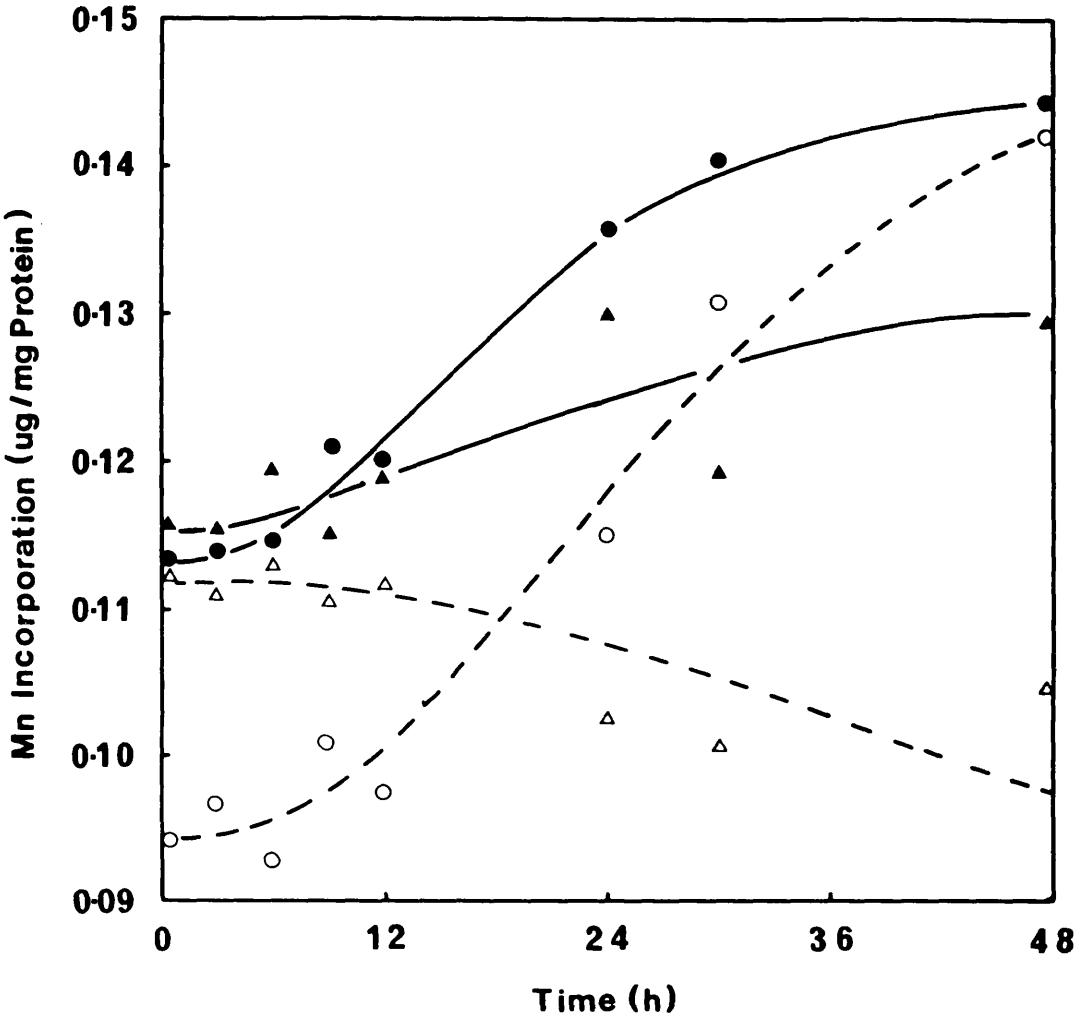


Figure 22. Graph showing the incorporation of Mn into chloroplast lamellae of greening pea explants, continuously illuminated over a period of 48h. Internal lamellae were isolated as described (methods 2.2). For Mn determination see Methods 5.5. (●) water/sucrose; (○) CMU/sucrose; (▲) water control; (△) CMU.

significant loss in manganese over the 48h illumination period.

The curves of each treatment time course were once again sigmoidal, and suggest the presence of a lag phase during the first 5 - 6h. The lag was short in the sucrose treatments but persisted in the substrate deficient water control. The lag was followed by a period of accelerated manganese uptake in all treatments other than the CMU treatment and may be related to the concomitant phase of active PS II development (see figures 20, 21).

4.2.3 PS I

The development of PS I as measured by ascorbate photo-oxidation is shown in figure 23. Onset of PS I activity occurred in all treatments other than the CMU treatment, after approximately 3 - 4h. Subsequent development of PS I was similar in these treatments and after a very short lag period, activities increased steadily to 24h. Both the H_2O and H_2O/sucrose treatments reached maximum rates at approximately 30h although the former was 33% higher. Subsequent illumination maintained steady state activities. In contrast the CMU/sucrose treatment increased further after 30h, and at 48h the activity was 65% and 35% above those of the respective H_2O and H_2O/sucrose treatments.

The ascorbate photooxidation assay for PS I is only useful for the determination of electron transport between plastocyanin and an artificial electron acceptor with a redox potential similar to ferredoxin (see figure 8). Additional information was provided by

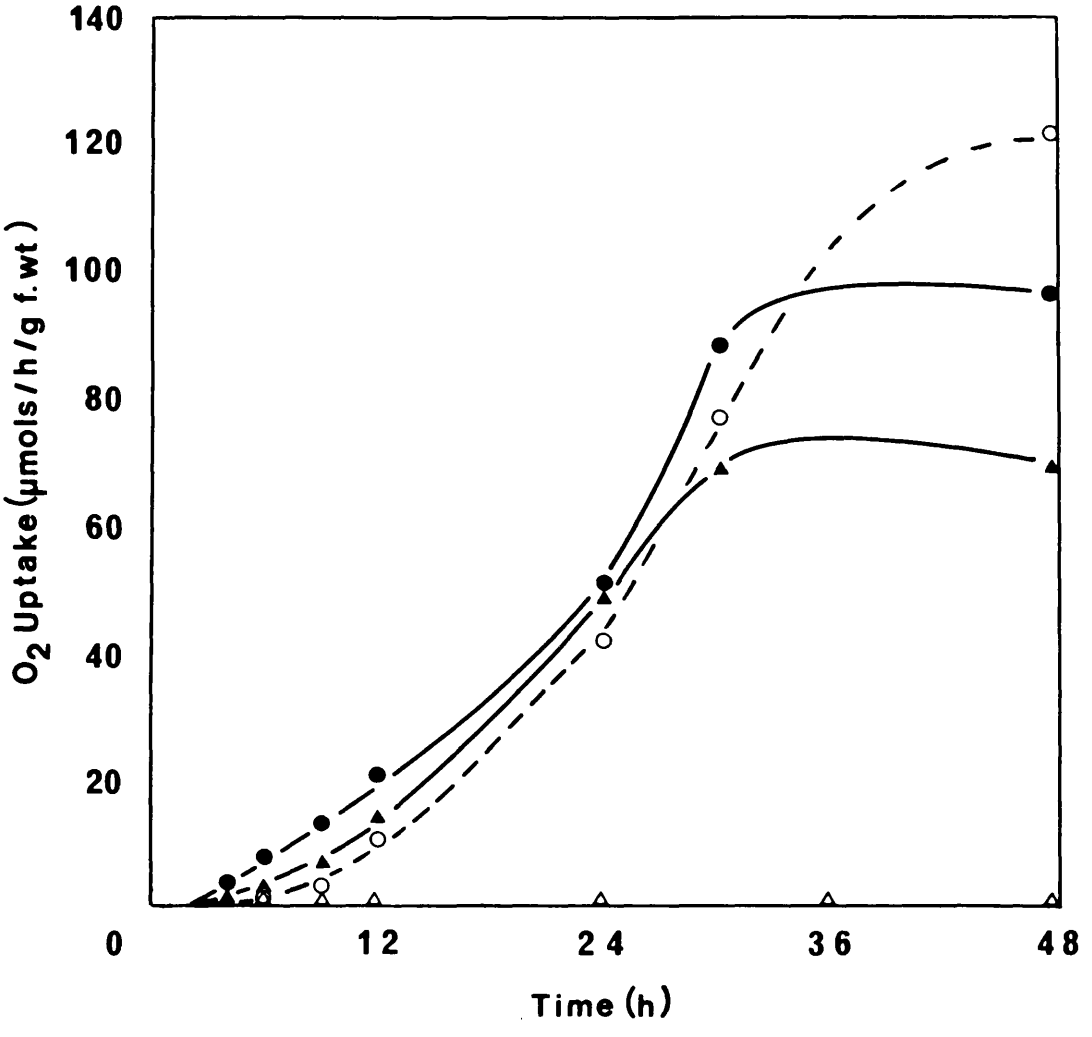


Figure 23. Time course showing the onset and development of PS I activity, as measured by ascorbate photo-oxidation, in plastids isolated from greening pea explants during 48h continuous illumination. O_2 uptake was measured in an O_2 electrode. (●) water/sucrose; (O) CMU/ sucrose; (▲) water control; (△) CMU.

initiating electron flow to the terminal electron acceptor $NADP^+$ through exogenously applied ferredoxin. PS I development determined by this method is shown in figure 24. Initial activity was detected in all treatments with the exception of the CMU treatment between 6 and 9h illumination. Subsequent development up to 24h was similar in both the water and water/sucrose treatments. By contrast the time course for the development of PS I activity in the CMU/sucrose treatment was parallel but substantially lower than the other treatments. Once again the development of the partial reaction described a sigmoidal plot and maximum rates were obtained at 30h. Steady rates or a slight fall off in rate occurred after this time, and it may be concluded from this and previous experiments that in the explant system the chloroplasts have reached maximum development at 30h illumination.

The development of PS I mediated cyclic phosphorylation catalysed by PMS is shown in figure 25. Activities were obtained from all treatments apart from that containing CMU alone. Chloroplasts isolated from water/sucrose treated plants produced the greatest activities which reached a maximum after 30h. Initiation of activity in both this and the CMU/sucrose treatment could not be detected before 12h but as these activities were high the point of initiation was extrapolated back to approximately 9h. Activity in the water treatment could not be detected before 18h illumination but this was again high and the assay was probably too insensitive to detect earlier activity. Cyclic phosphorylation activity in chloroplasts isolated from greening Phaseolus vulgaris has been detected at 5h (Dodge and Whittingham, 1966; Whatley and Gyldenholm, 1968) and 4h in barley chloroplasts

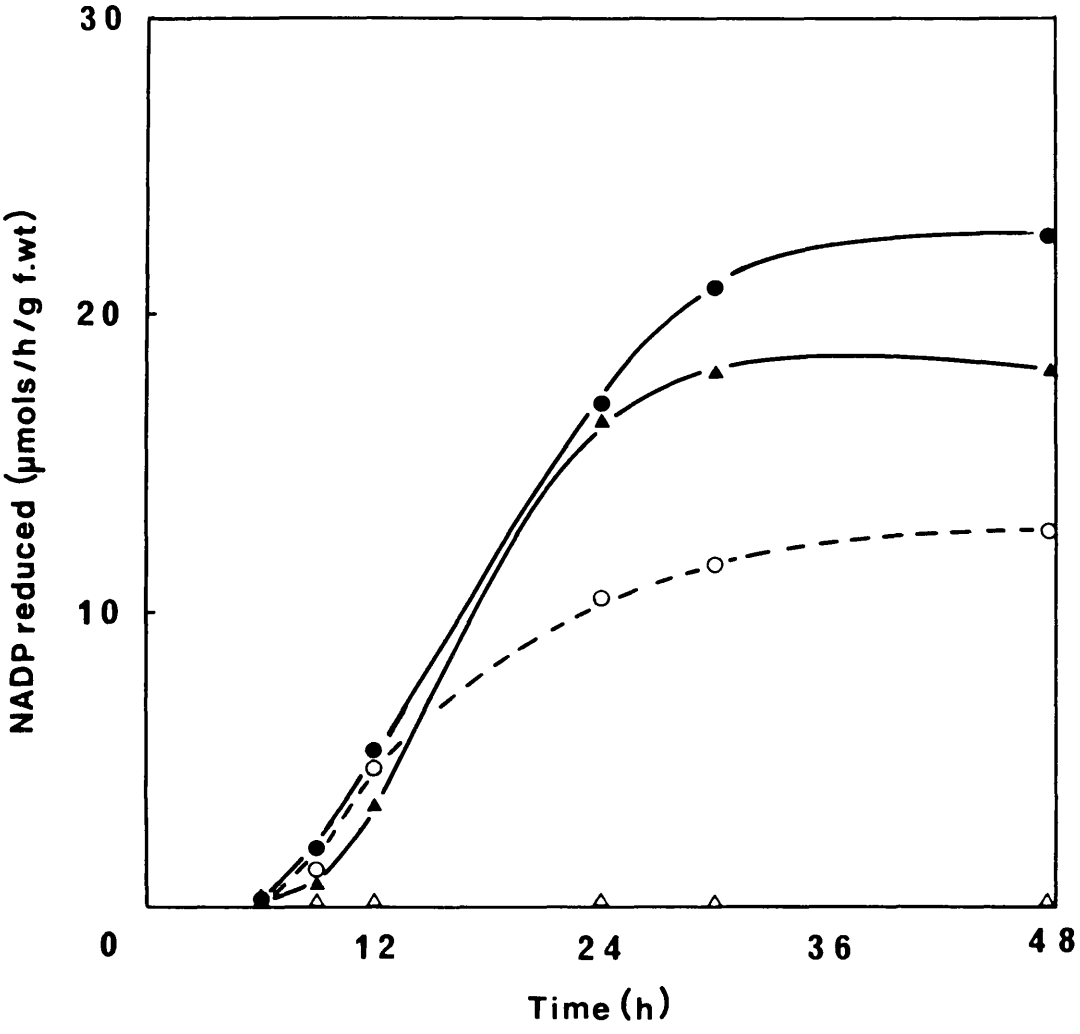


Figure 24. Time course showing the development of PS I, as measured by $NADP^+$ reduction, in plastids isolated from greening pea explants during 48h continuous illumination. $NADP^+$ reduction was measured spectrophotometrically (see Methods 3.2.1). (●) water/ sucrose; (○) CMU/sucrose; (▲) water control; (△) CMU.

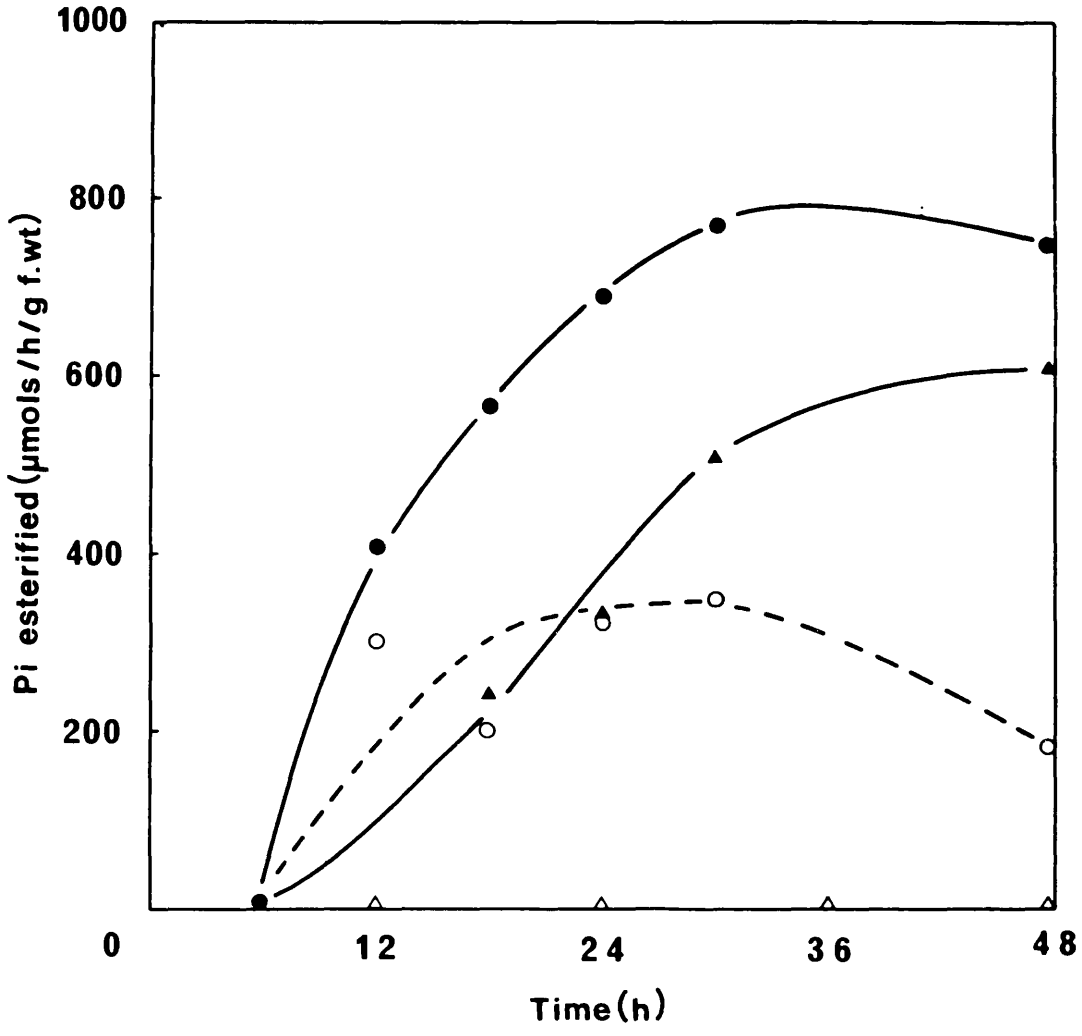


Figure 25. Time course showing the development of PMS-catalysed cyclic phosphorylation in plastids isolated from greening pea explants over 48h continuous illumination. Experimental procedure and phosphate determinations were carried out as described in Methods 3.3 and 5.2 respectively. (●) water/sucrose; (○) CMU/sucrose; (▲) water control; (△) CMU.

(Phung-Nhu-Hung et al, 1970 a).

The subsequent development of cyclic phosphorylation in the water control was approximately parallel to the H_2O/sucrose treatment but at about 50% of the rate. Initial activities of the CMU/sucrose treatment up to 24h illumination were above those of the water treatment. Subsequent development, however, appeared to be impaired and after 48h the activity of this treatment was only 25% of the H_2O/sucrose treatment and 33% of the H_2O treatment.

5. STRUCTURAL DEVELOPMENT

5.1 Development of lamellae chlorophyll-protein complexes

Isolated SDS solubilised chloroplasts electrophoresed on polyacrylamide gel for 2h resolve into three major pigment bands (Thornber et al, 1966). In addition to these bands two less intense bands were also observed. Figure 26A shows a densitometer scan at 660 nm of an unstained gel. Bands 2, 10 and 18 represent the three major pigmented bands and bands 1 and 3 the less intense minor bands. Although pigmented band 10 appears to be small, figure 27 shows that band 18 increased at the expense of the former during the time of electrophoresis. This evidence is consistent with that of Thornber (1967) although contradictory evidence has suggested that the movement of chlorophyll to band 18 is in fact from a second complex which runs concurrently with band 10 (Genge et al, 1974). Figure 27 also shows

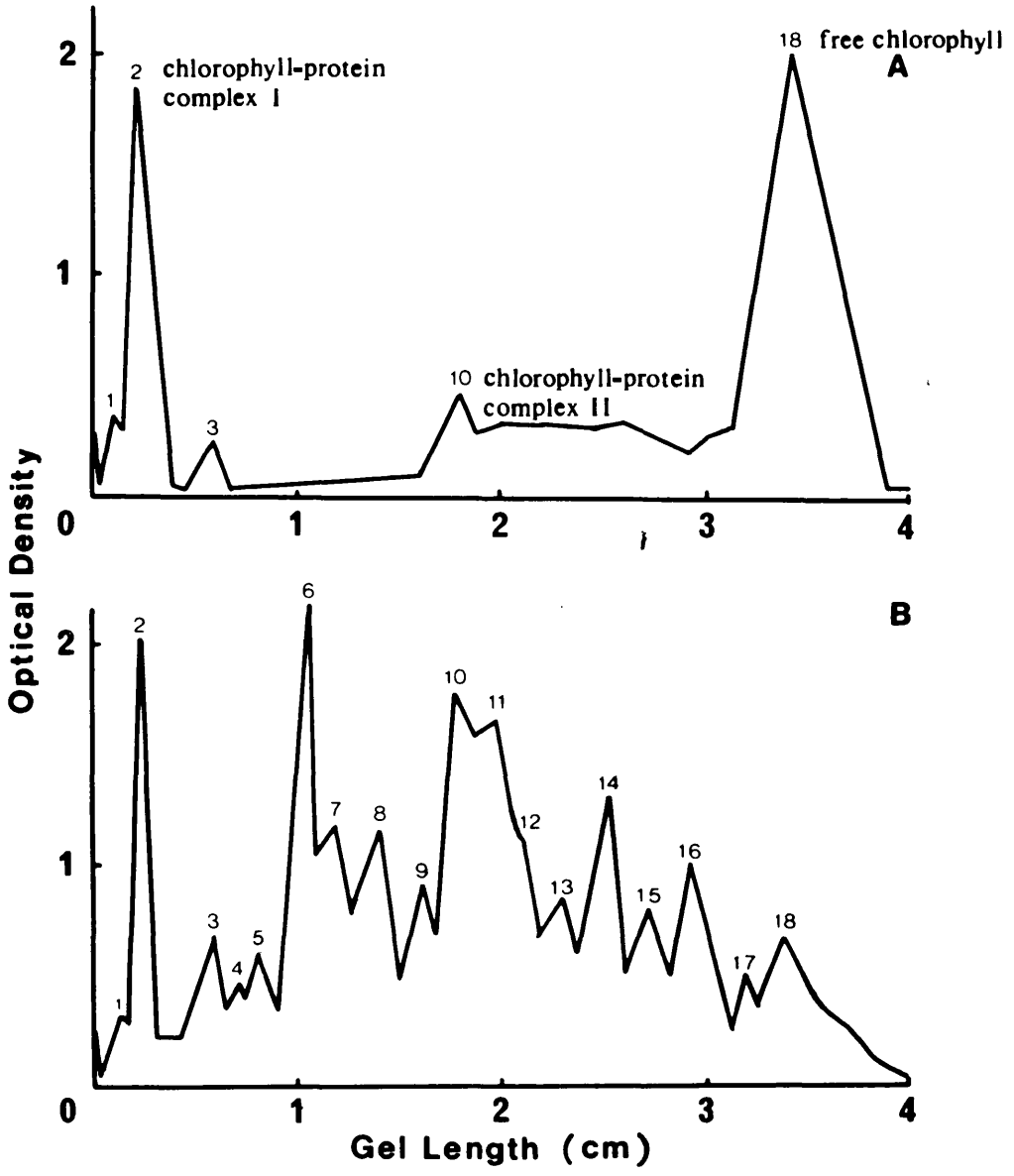


Figure 26. A. Densitometer scan at 660 nm of solubilised chloroplast lamellae, resolved by SDS polyacrylamide gel electrophoresis. Lamellae were isolated from water/sucrose treated pea explants, greened for 48h. Protein (40µg) was applied to pre-electrophoresed 15% SDS polyacrylamide gels and electrophoresed at 4 mA/gel for 2h.

B. Densitometer scan at 540 nm of identically treated polyacrylamide gels, stained for protein with 0.75% coomassie brilliant blue R 250 subsequent to electrophoresis.

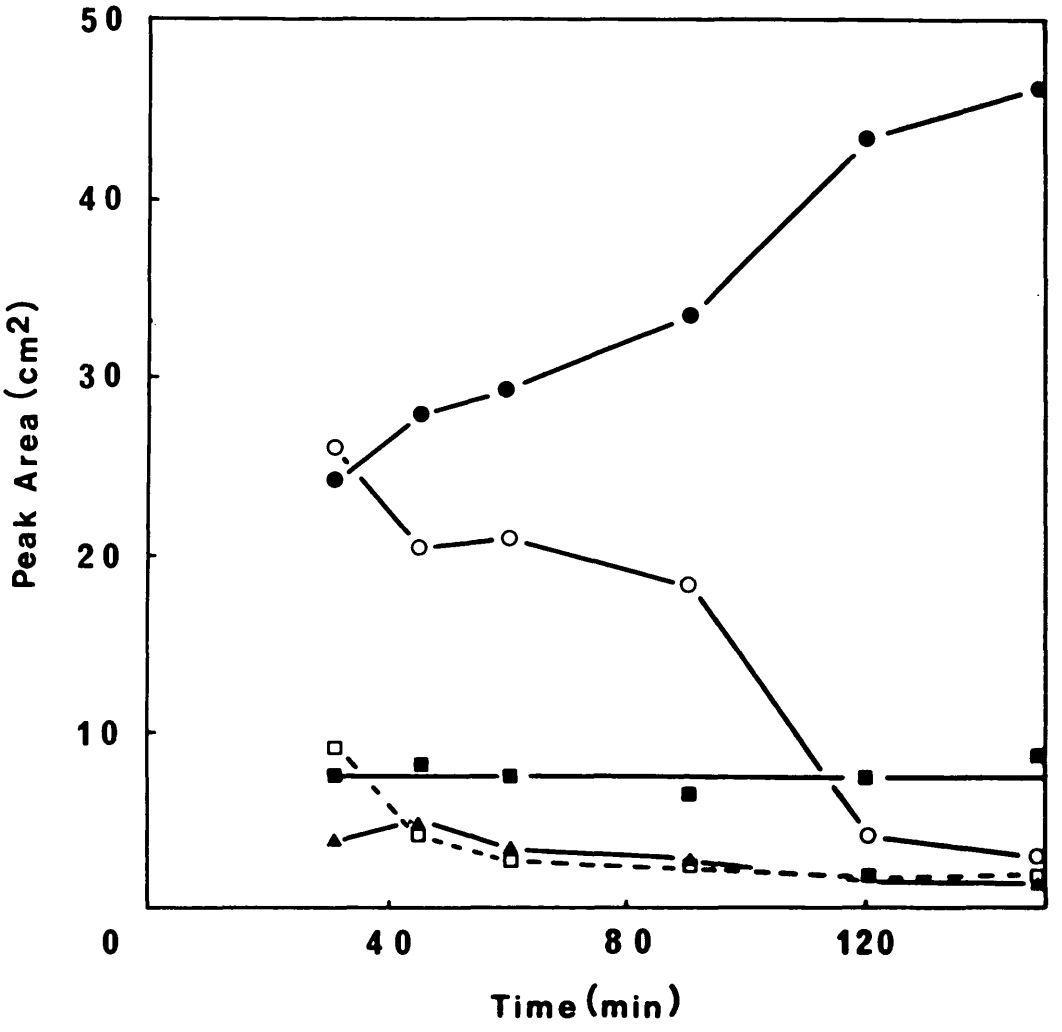


Figure 27. Changes occurring in the chlorophyll content of the chlorophyll-protein complexes during electrophoresis. (●) free chlorophyll (band 18); (○) chlorophyll-protein complex II (band 10); (■) chlorophyll-protein complex I (band 2); (▲) band 3; (□) band 1. Pre-electrophoresed 15% SDS polyacrylamide gels were loaded with 40ug of solubilised chloroplast lamellae from water/sucrose treated explants and electrophoresed at 4 mA/gel for 2½h. Gels were removed at intervals during the run and scanned at 660 nm.

that little pigment is removed from band 10 until after 90 min electrophoresis. The reason for the removal of this bound chlorophyll is uncertain but could possibly be as a result of the heat generated during electrophoresis. Bands 1 and 3 may also contribute to band 18 since a small amount of chlorophyll was also lost during electrophoresis. Band 2, however, remained unchanged.

Staining of an identically electrophoresed gel with the protein stain coomassie blue, revealed the resolved protein content of the chloroplast membranes. A densitometer scan of the gel at 540 nm is shown in figure 26B. Each band is numbered in increasing order of mobility and the bands of the corresponding unstained gel (figure 26A) have the same number as the corresponding protein band. Two of the three pigmented bands (2 and 10) therefore correspond to major protein bands, and these have been designated chlorophyll-protein complexes I and II respectively (Thornber et al., 1966). Band 18 contains no protein and is generally referred to as the free chlorophyll band. Bands 1 and 3 are also minor complexes and it is thought these are polymers of the two major complexes (Thornber, 1975).

5.1.1 Chlorophyll-protein complex II

Spectral studies on the pigment composition of chlorophyll-protein complexes I and II have shown them to be associated with PS I and II respectively. Hence the results that follow are a study of the changes occurring in these chlorophyll-protein complexes during greening over the selected 48h illumination period.

Gel traces were quantified by measuring the area under each peak. It is assumed that the protein loading used is within the linear limits of coomassie blue protein stain.

Changes in the protein associated with chlorophyll-protein complex II (Band 10) in plastids isolated from plants treated with and without CMU and sucrose, are shown in figure 28. The protein was present in small amounts in etioplasts. In all treatments it was shown to increase independently of both photosynthesis and chlorophyll synthesis. No change in the protein was shown at 3h and subsequent increases were small and similar in all treatments up to 12h. Following the lag period a phase of active protein synthesis ensued and between 12 and 30h a twelve fold increase in the content of this protein was observed in the treatments containing sucrose. The rates decreased slightly after 30h but at 48h the protein content had increased by a further 22%. Smaller increases occurred in the protein of the H_2O and CMU treatments. After 30h the content was approximately four fold higher than that at 12h. The water treatment, however, followed the trends of the H_2O/sucrose and CMU/sucrose treatments already mentioned but no further increase was observed in the CMU treatment.

5.1.2 Changes occurring in chlorophyll-protein complex I (Band 2)

Changes occurring in chlorophyll-protein complex I during illumination followed closely those of chlorophyll-protein complex II (figure 29). Once again small quantities of the protein were present in the prolamellar bodies of the etioplasts and were unaffected by the

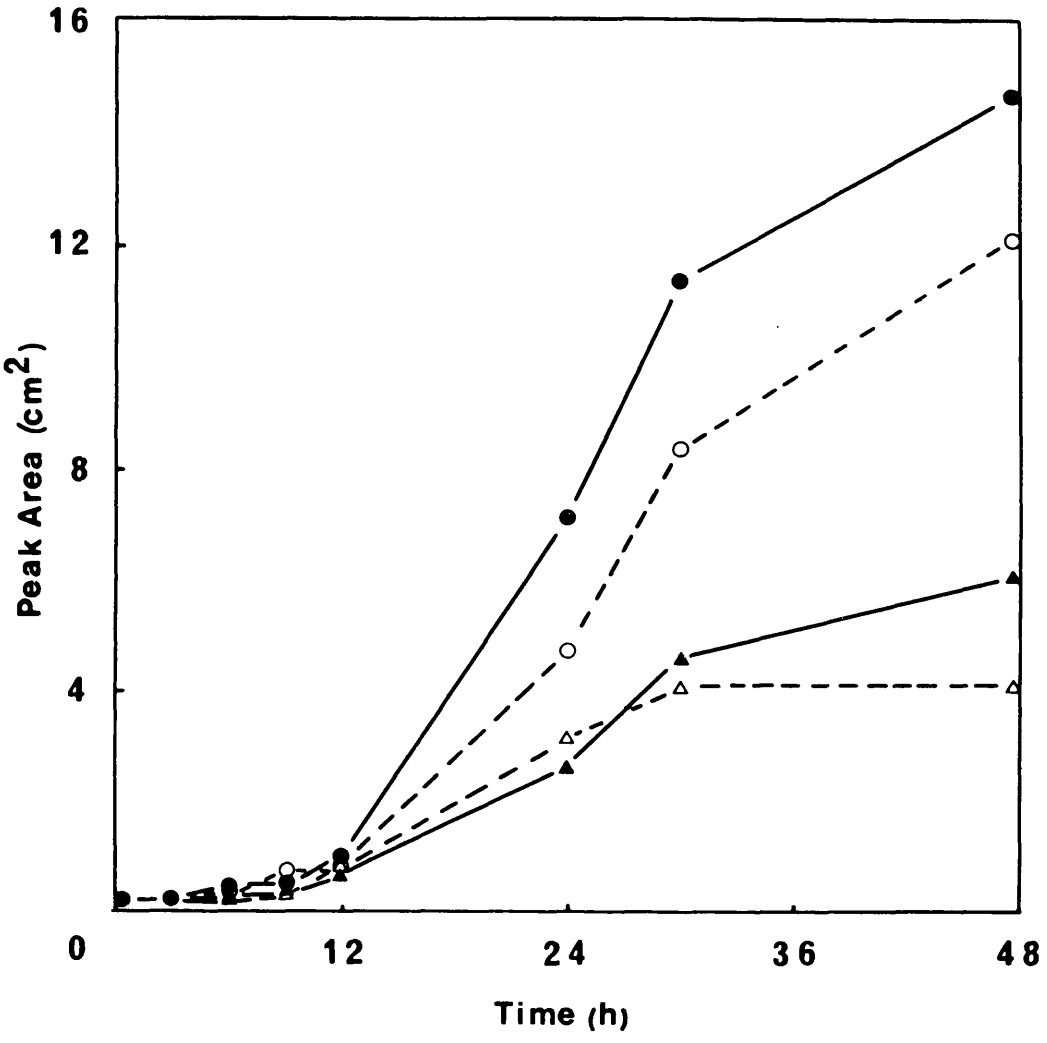


Figure 28. Changes occurring in the protein associated with chlorophyll-protein complex II during the greening of pea explants over 48h continuous illumination. Lamellae were isolated, solubilised and resolved by SDS polyacrylamide gel electrophoresis as described in Methods 2.3 and 7 respectively. (●) water/sucrose; (○) CMU/sucrose; (▲) water control; (△) CMU.

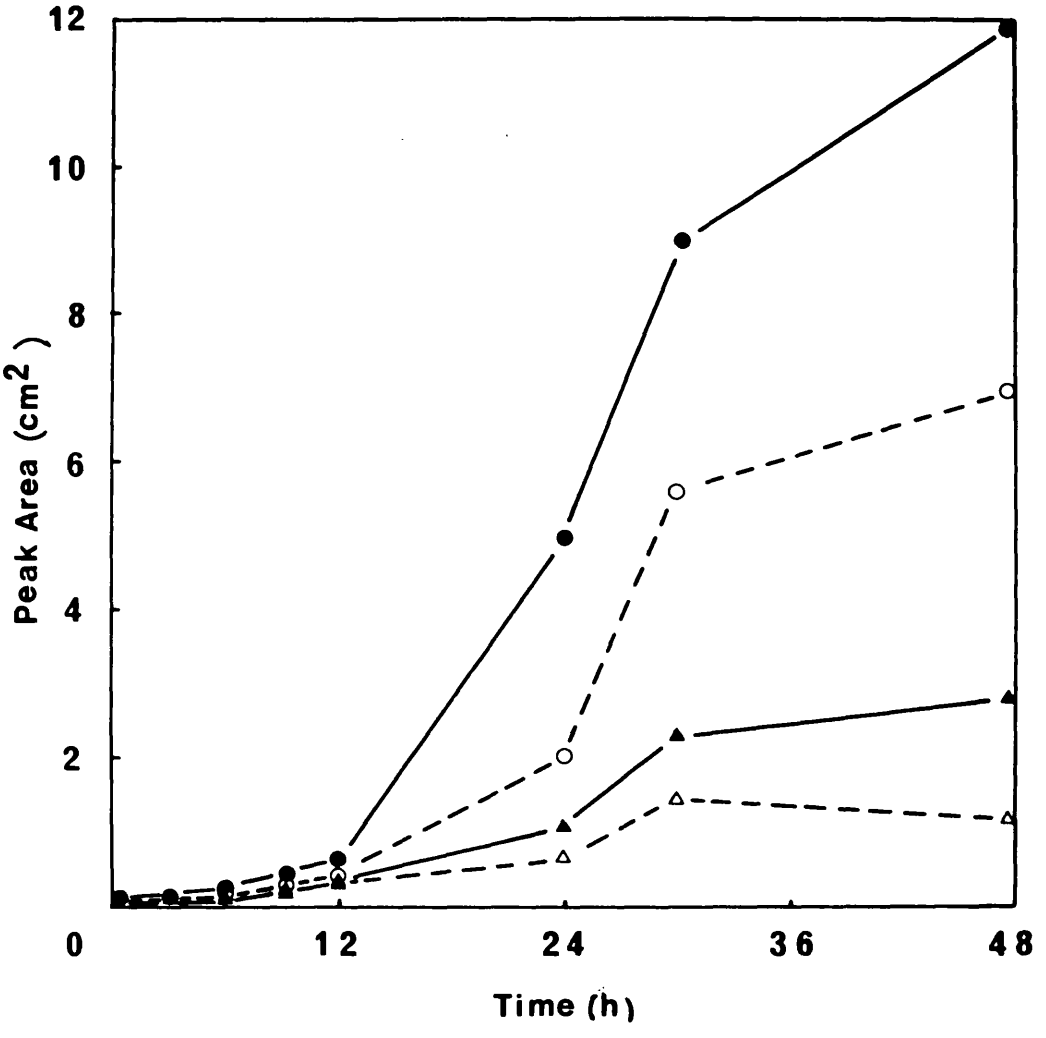


Figure 29. Changes occurring in the content of the protein associated with chlorophyll-protein complex I during the greening of pea explants during 48h continuous illumination. Lamellae were isolated, solubilised and resolved by SDS polyacrylamide gel electrophoresis as described in Methods 2.3 and 7 respectively. (●) water/sucrose; (○) CMU/sucrose; (▲) water control; (△) CMU.

plant treatment prior to illumination. The lag phase occurring in chloroplast development during the first few hours of illumination was general to all treatments, up to 6h. Subsequent illumination produced a gradual but approximately linear increase in protein contents associated with the plastids of the water controls. In the CMU treated explants the increase was considerably lower but nevertheless significant up to 30h after which point no further increase occurred. Substitution for photosynthesis by the addition of sucrose to the CMU treated explants produced large increases in the protein complex with a two fold increase in content above that of the water control after 48h. The greatest increase in chlorophyll-protein complex I content was once again produced by the plastid of the water/sucrose treated explants.

5.1.3 Changes in minor band I

In addition to chlorophyll-protein complexes I and II minor chlorophyll containing bands 1 and 3 are also complexed with protein. The presence of band I protein cannot be detected in plastids isolated from the water control and CMU treatments, during the entire period of illumination, and is only present in the two treatments containing sucrose at 24h (figure 30). After this time and up to 48h the protein was observed to significantly increase in content in the water/sucrose treatment. In contrast the protein detected at 24h in the CMU/sucrose treatment was only seen to increase up to 30h. A subsequent decrease in content was produced in response to further

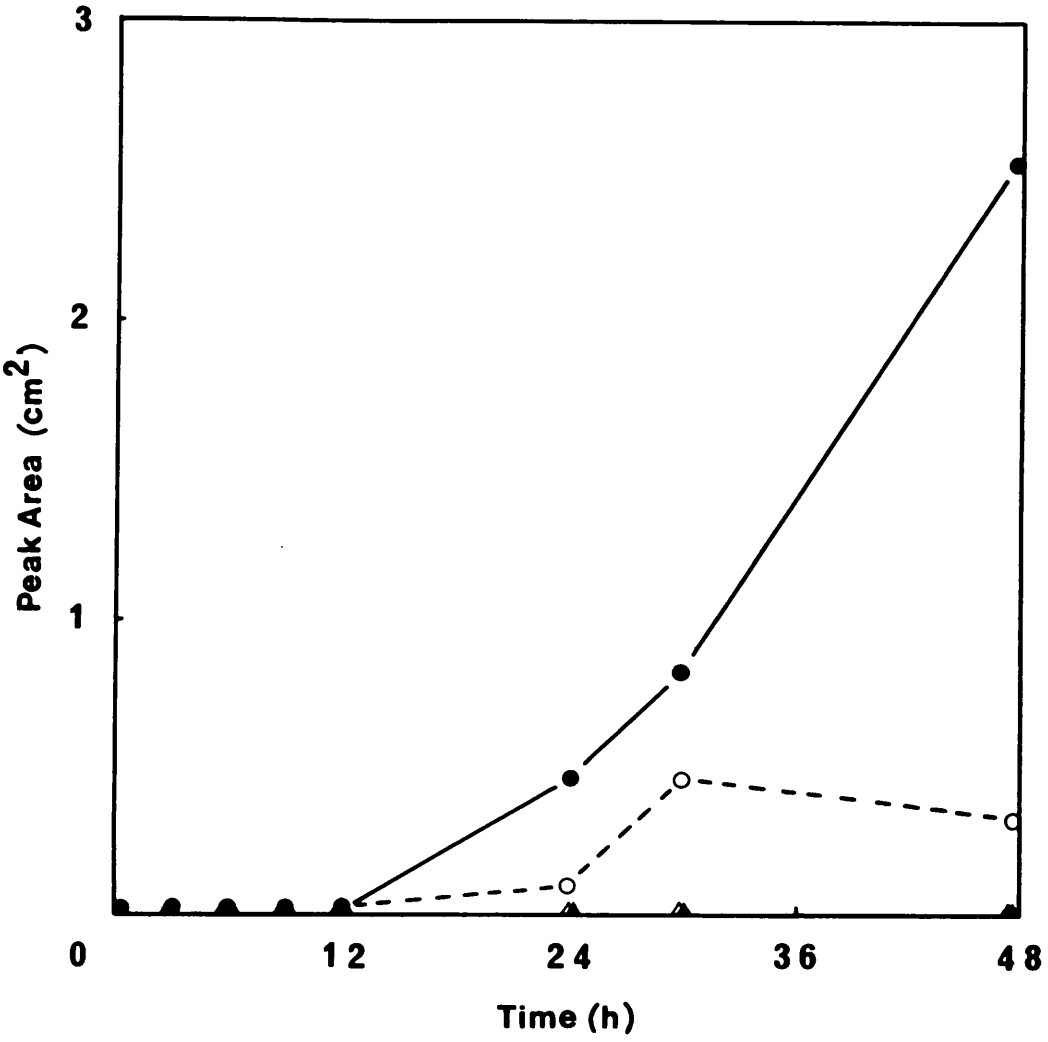


Figure 30. Changes occurring in the content of the protein complex (band 1) during greening of pea explants over a period of 48h continuous illumination. (●) water/sucrose; (○) CMU/sucrose; (▲) water control; (△) CMU.

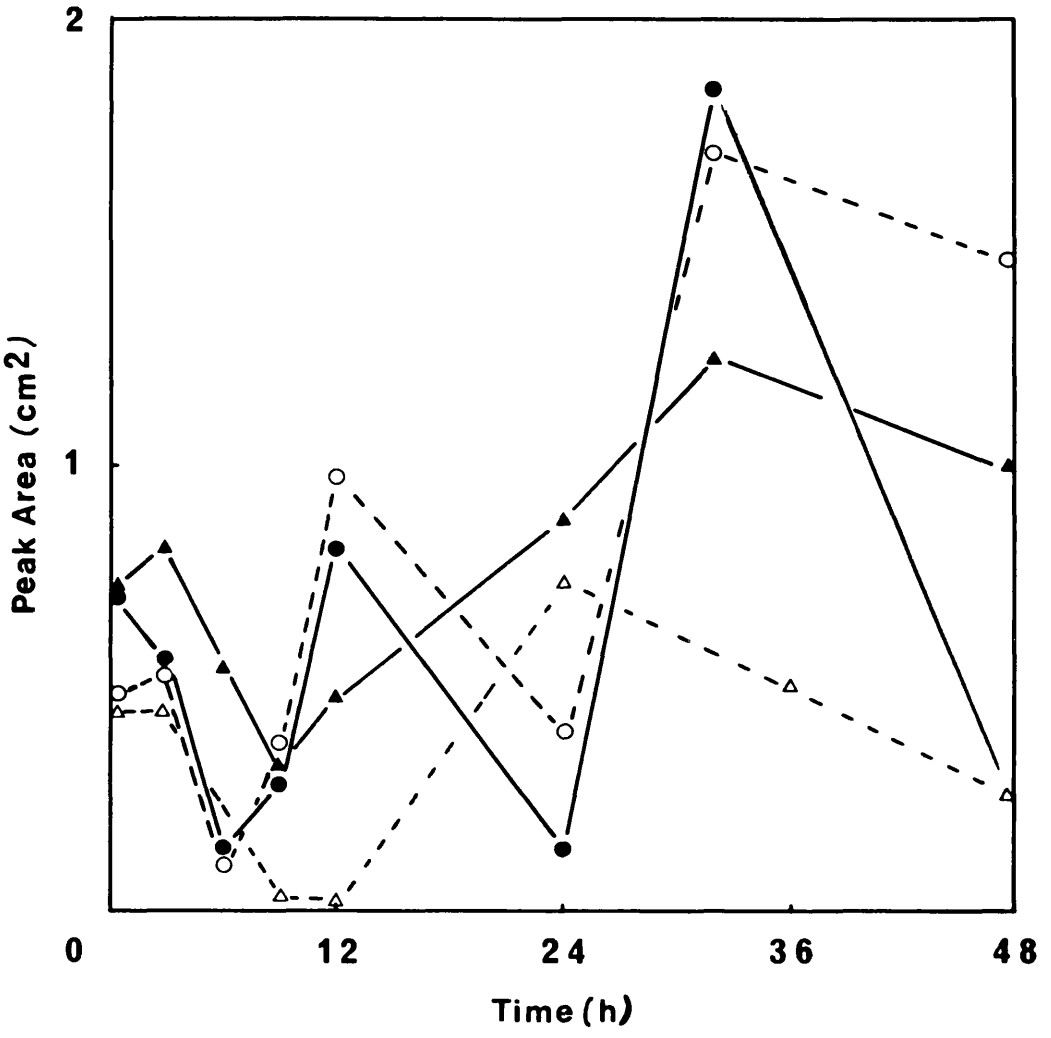


Figure 31. Changes occurring in the content of the protein associated with the minor chlorophyll-protein complex (band 3) during the greening of pea explants over 48h continuous illumination. (●) water/sucrose; (○) CMU/sucrose; (▲) water control; (△) CMU.

illumination.

5.1.4 Band 3

The changes occurring in the protein content of the minor chlorophyll-protein complex (band 3) are more confusing (figure 31), as large fluctuations were observed in each of the four treatments. However certain generalisations can be made from the graph. This protein was present in etiolated plastids at concentrations higher than those of the chlorophyll-protein complex I but approximately equal to those of complex II. During the first 6 - 9h of illumination the content of the protein after an initial small increase, was observed to decrease in each of the treatments and therefore was independent of photosynthesis. After 9h illumination the content increased steadily in the water control treatment up to 30h and then decreased during the final 18h. General increases were also observed in the water/sucrose and CMU/sucrose treatments although large fluctuations occurred at 24h. Band 3 protein also increased after an initial decrease between 3 and 12h in the CMU treated leaves. Maximum content was after 24h after which a decrease was observed.

5.2 Changes occurring in chloroplast ultrastructure

The development of chloroplast fine structure over the 48h illumination period and for each of the four treatments is shown in plates 1 - 33. Each micrograph has been chosen to be representative

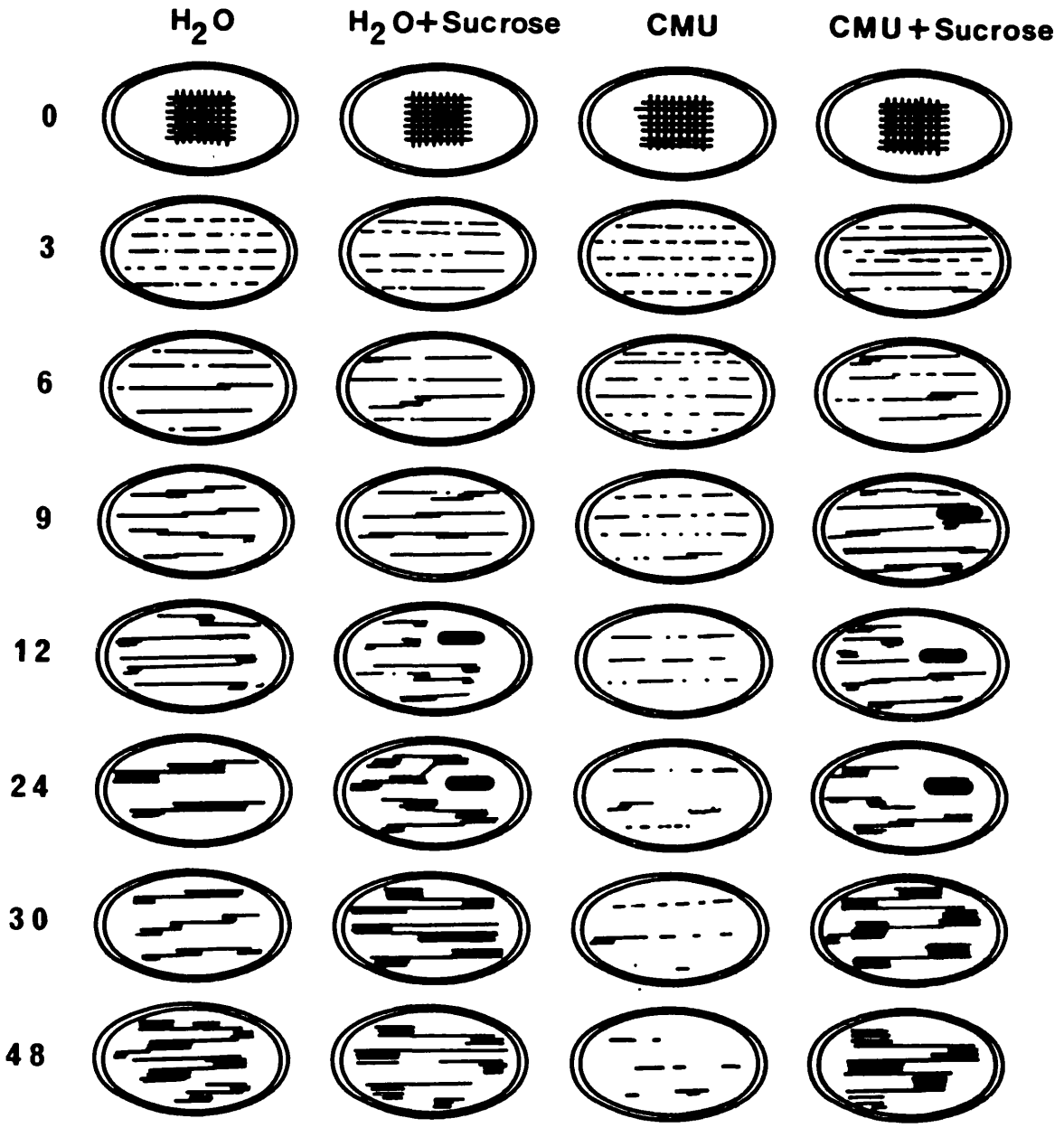


Figure 32. Schematic representation to summarise the changes occurring in chloroplast fine structure during greening in pea explants over a period of 48h. Actual changes are shown by the electron-micrographs in plates 1 - 34. ## - prolamellar body; ---- perforated thylakoid; ——— primary thylakoid; granum; disorganised granum; starch grain

of the particular stage of chloroplast development as variations in structure can occur between two adjacent plastids in the same tissue. In some instances it has been found necessary to show more than one micrograph for a particular time stage. In addition a diagrammatic scheme has been drawn (figure 32) in which the developmental chloroplast changes have been schematized.

Normal development of a mature chloroplast from an etioplast is shown in the micrographs obtained from leaf sections of the water control explants. This followed closely the observations of other workers (Virgin et al, 1963; Klein et al, 1964).

The starting material (8 day old etiolated explants) contained etioplasts as shown in plate 1. The prolamellar body was clearly visible and so were a number of lamellae radiating out from this structure. Whereas the prolamellar body was common to all etioplasts the number and length of the radiating lamellae varied from plastid to plastid. Klein (1960) suggested that the protrusion of vesicles from the prolamellar body could occur in response to the green light used during manipulation of the plant material.

In leaves which were exposed to light of 4 W/m^2 for 3h the development of fine structure had progressed to the stage where the prolamellar bodies had dispersed (Virgin et al, 1963) into perforated thylakoids arranged in rows or occasionally rings (plate 2). Little or no thylakoid stacking at this stage was observed .

After 6h illumination (plate 3) the perforated thylakoids were disappearing and featureless primary thylakoids were forming in their place (Kirk and Tilney-Bassett, 1967). In addition, a few areas

of grana formation appeared, containing no more than two thylakoids. After 9h the plastids of these control leaves contained a larger number of appressed lamellae and small grana consisting of 4 - 5 thylakoids (plate 4). No marked changes were observed after a further 3h illumination although the number of grana per plastid containing four to five membranes had increased (plate 5). The length of each grana stack had also increased, thus producing a greater surface area of appressed lamellae.

At 24h (plate 6) the area of grana per plastid of the control leaves had considerably increased. Once again the number of thylakoids contained in each granum was no greater than at the earlier developmental stages, but the frequency and length of the grana had more than doubled. A further six hours produced an even greater increase in grana content per plastid and in addition many of the grana consisted of 5 - 7 thylakoids (plate 7). After 48h illumination many of the grana contained 6 - 8 thylakoids and were considerably longer than at previous stages of development (plate 8). This chloroplast developmental sequence demonstrated by the control plants was dependent upon photosynthesis for required substrate. Between 0 - 6h illumination changes which occurred in the internal membrane were thought to be only a rearrangement of membrane stored within the prolamellar body (Gunning and Jagoe, 1965; Henningsen and Boynton, 1969). Subsequent illumination, particularly between 12 and 48h, produced a vast increase in new membrane and this was attributed to substrate production after photosynthetic competence was achieved.

Changes occurring in the etioplasts of plants treated with

the photosynthetic inhibitor CMU, are shown in plates 9 to 18 and in figure 33. The etioplast shown in plate 9 contains a large central prolamellar body from which there are few radiating lamellae and which also displays a good crystalline structure. After 3h (plate 10) illumination the prolamellar body had dispersed to produce perforated thylakoids and no significant differences could be seen between the plastids of this treatment and those of the water control. At 6h (plate 11) however little change had occurred in the development of these plastids. The perforated thylakoids persisted and there was little grana formation, although what appears to be the beginning of appressed lamellae can be observed in places. Further illumination produced little effect and after 9h (plate 12) there was a negligible difference between these plastids and those of the earlier illumination times.

After 12h illumination (plate 13) grana formation was not visible and there was a noticeable decline in the lamellae content of the plastids. Over the 36h illumination period which followed, the lamellae content gradually decreased (plates 14, 15 and 16). It may be concluded therefore that without a source of substrate chloroplast development cannot continue beyond the stage of prolamellar body dispersal. It is evident that grana formation requires the synthesis of new membrane material (Heslop-Harrison, 1966; Kirk and Tilney-Bassett, 1967).

The effects of adding exogenous sucrose to CMU treated plants on the fine structural development is shown in plates 17 to 25. The development of the chloroplast in CMU/sucrose treated pea explants was

not significantly different from that of the controls. Between 0 and 3h illumination, the period which is considered to be the lag phase in chloroplast development, the prolamellar body dispersed to form perforated thylakoids (plate 18). At 6h a small number of small grana were present, each consisting of not more than three thylakoids (plate 19). Further illumination produced rapid grana formation and at 9h many small grana consisting of up to four thylakoids, were present (plate 20). Also present at 9h (plate 21) were considerable amounts of starch as shown by the conspicuous starch grains. Between 9 and 12h illumination (plate 22) the thylakoid content again increased by lengthening of the grana stacks. Starch was present at this time but not in such large quantities. Illumination up to 30h produced a vast increase in quantity and size of grana stacking (plates 23 and 24). Further illumination, however, appeared to produce deleterious effects on grana integrity as shown by swelling of the individual thylakoids after 48h (plate 25).

The presence of an endogenous substrate in photosynthetically competent explants produced a developmental sequence not dissimilar to that of the water control (plates 26 to 33). A lag phase lasting approximately 6h, during which the prolamellar body dispersed to form perforated and primary thylakoids, and during which few grana were formed, was followed by a period of active grana formation. During this latter period, plastid development at any stage was more advanced than in the water control. This was shown by quantity and size of the grana stacks which regularly consisted of between 5 and 12 thylakoids. The formation of starch grains was observed as early as 12h although

these were probably formed from the exogenous sucrose rather than by substrate from chloroplast photosynthesis. Greater quantities of starch were again seen at 24h, although this was completely utilised or diminished by 30h. As in the CMU/sucrose treatment loss of granum integrity occurred between 30 and 48h illumination as demonstrated by the swelling of the thylakoids within the grana stacks.

Figure 34 has been added to show the initial changes which occurred in the fine structure of the prolamellar body after 10 min illumination. A loss in the crystallinity of the prolamellar body was demonstrated (see Introduction section 1.3) without any significant dispersal or rearrangement of the lamellae.

Plates 1 - 8: Changes occurring in the fine structure of chloroplasts in water control leaves during 48h illumination. Leaf sections were pre-fixed with glutaraldehyde, post-fixed with osmium tetroxide and embedded in standard spur (for details see Materials and Methods section 8).

Plate 1: Oh water control Magnification x 51,330

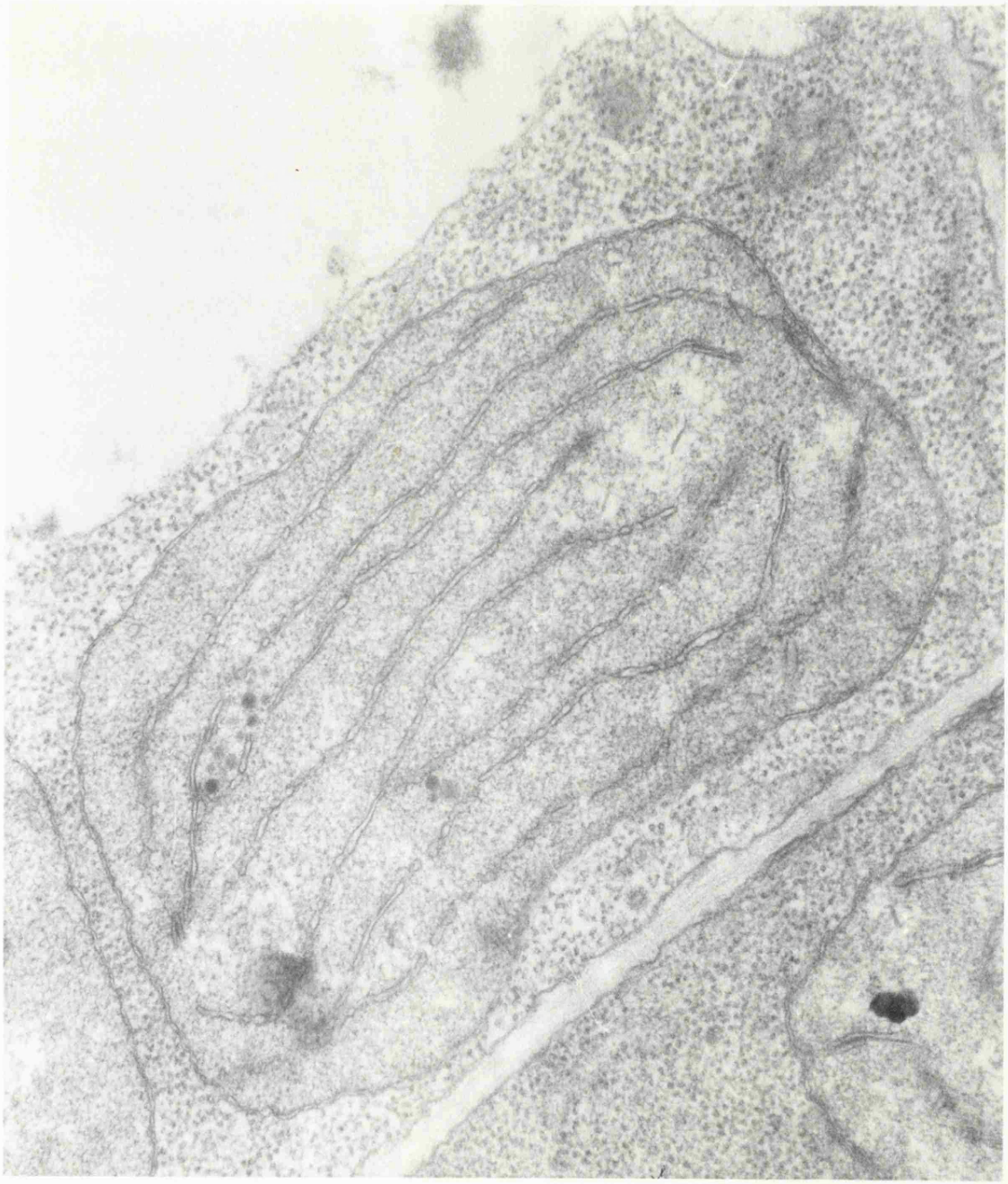


Plate 2: 3h water control Magnification x 51,330

Plate 3: 6h water control Magnification x 51,330

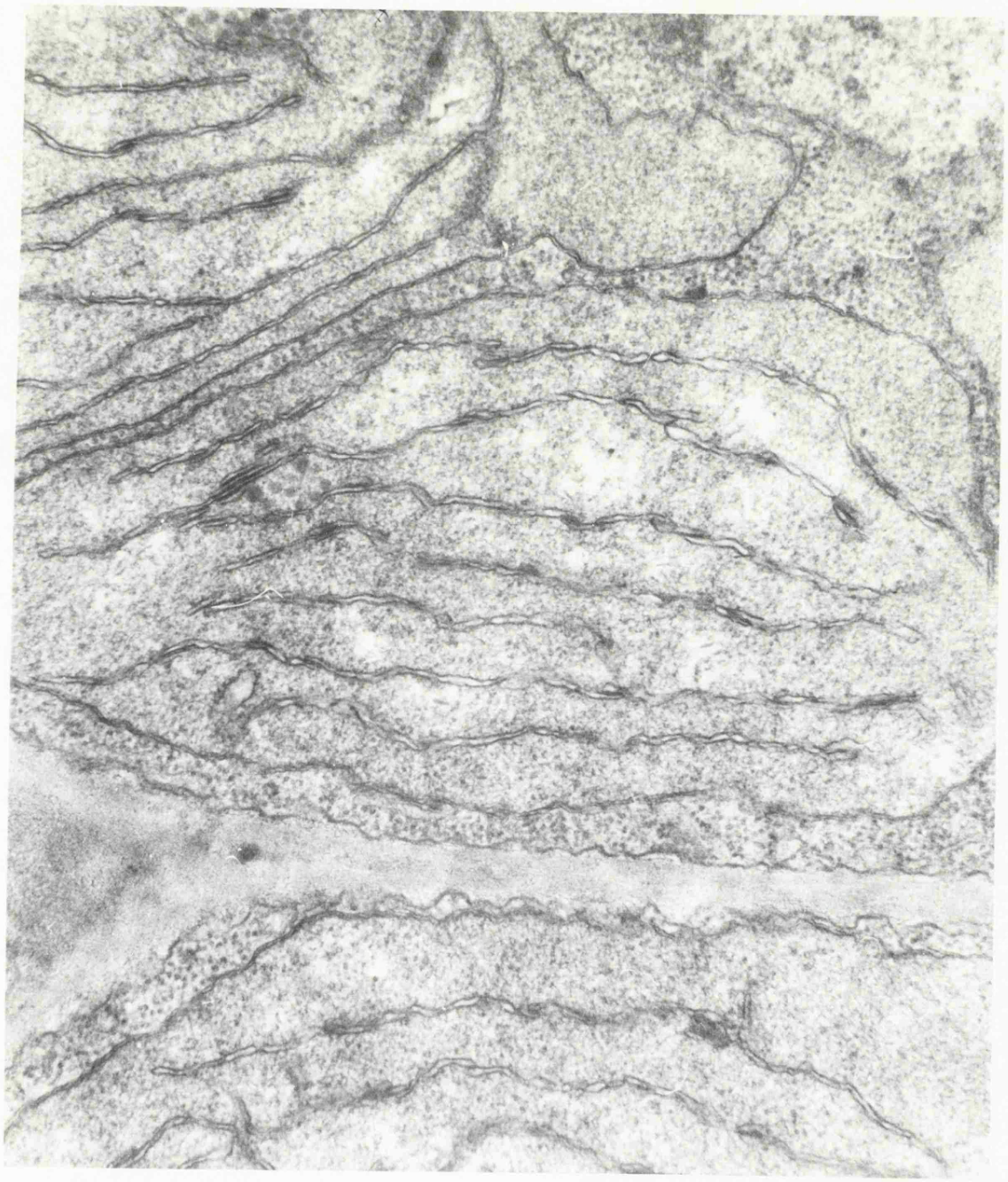


Plate 4: 9h water control Magnification x 51,330

Plate 5: 12h water control Magnification x 51,330

Plate 6: 24h water control Magnification x 51,330

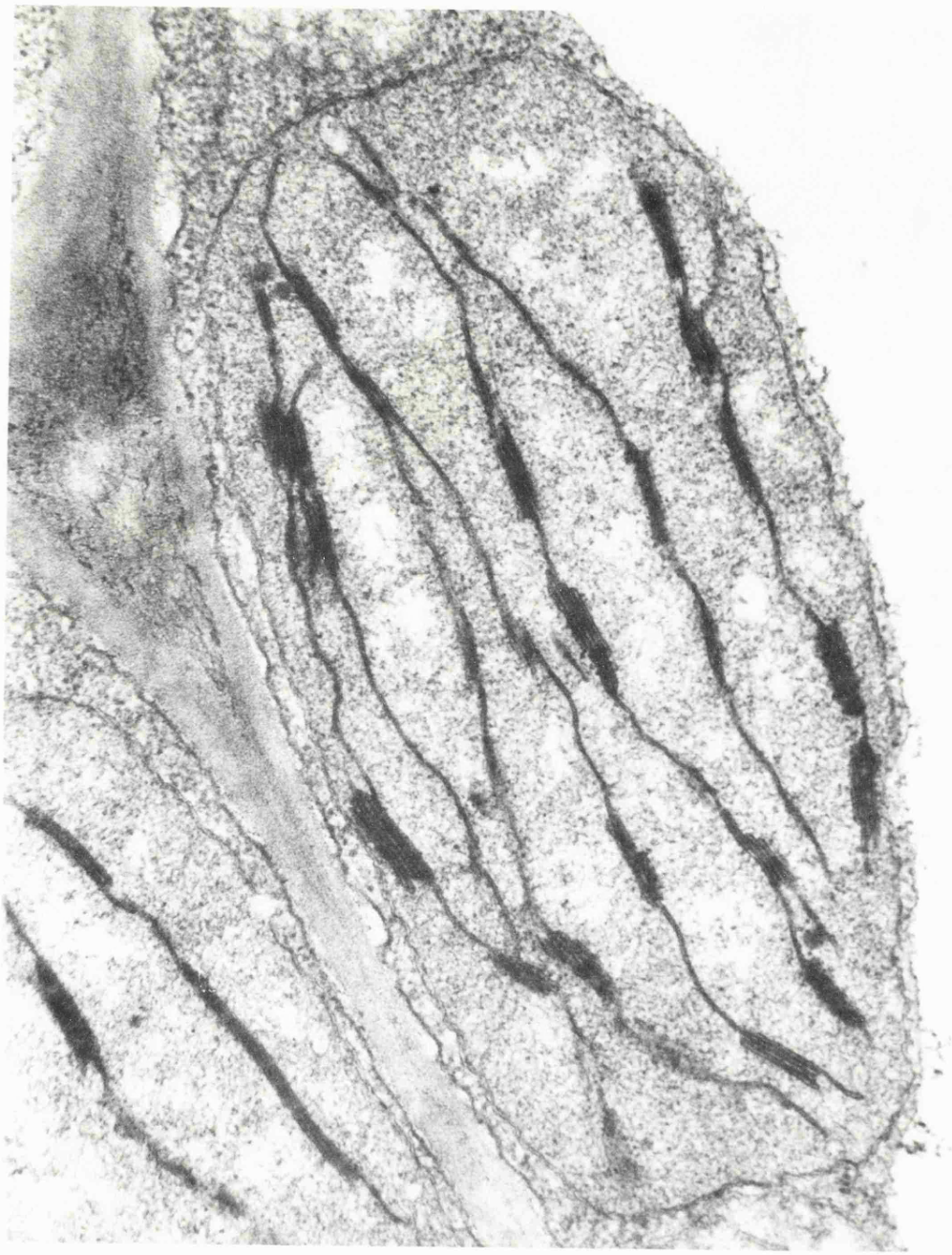


Plate 7: 30h water control Magnification x 51,330

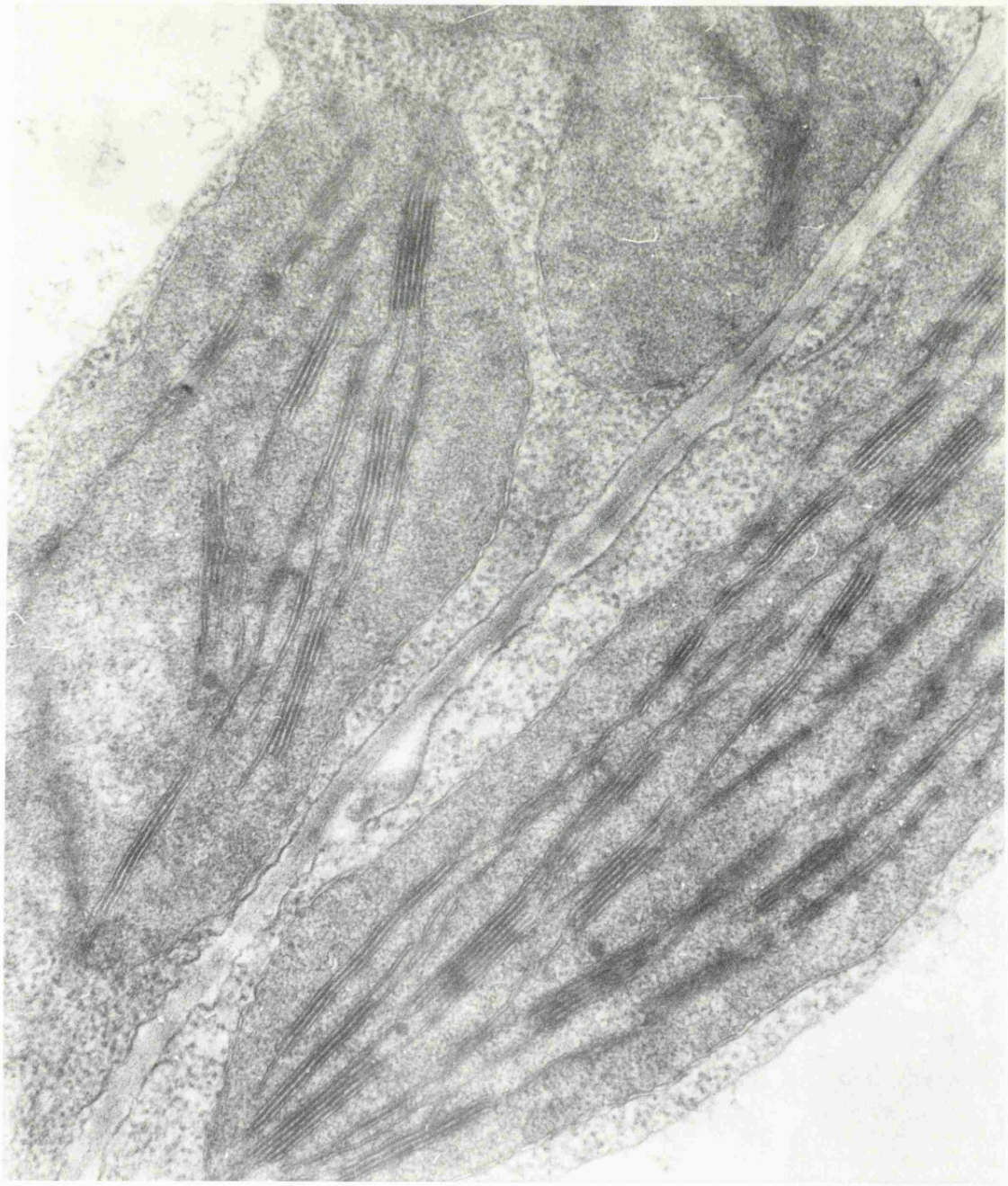


Plate 8: 48h water control Magnification x 51,330

Plates 9 - 16: Changes occurring in the fine structure of chloroplasts in leaves treated with CMU during 48h illumination. Leaf sections were pre-fixed in glutaraldehyde, post-fixed in osmium tetroxide and embedded in standard spur.

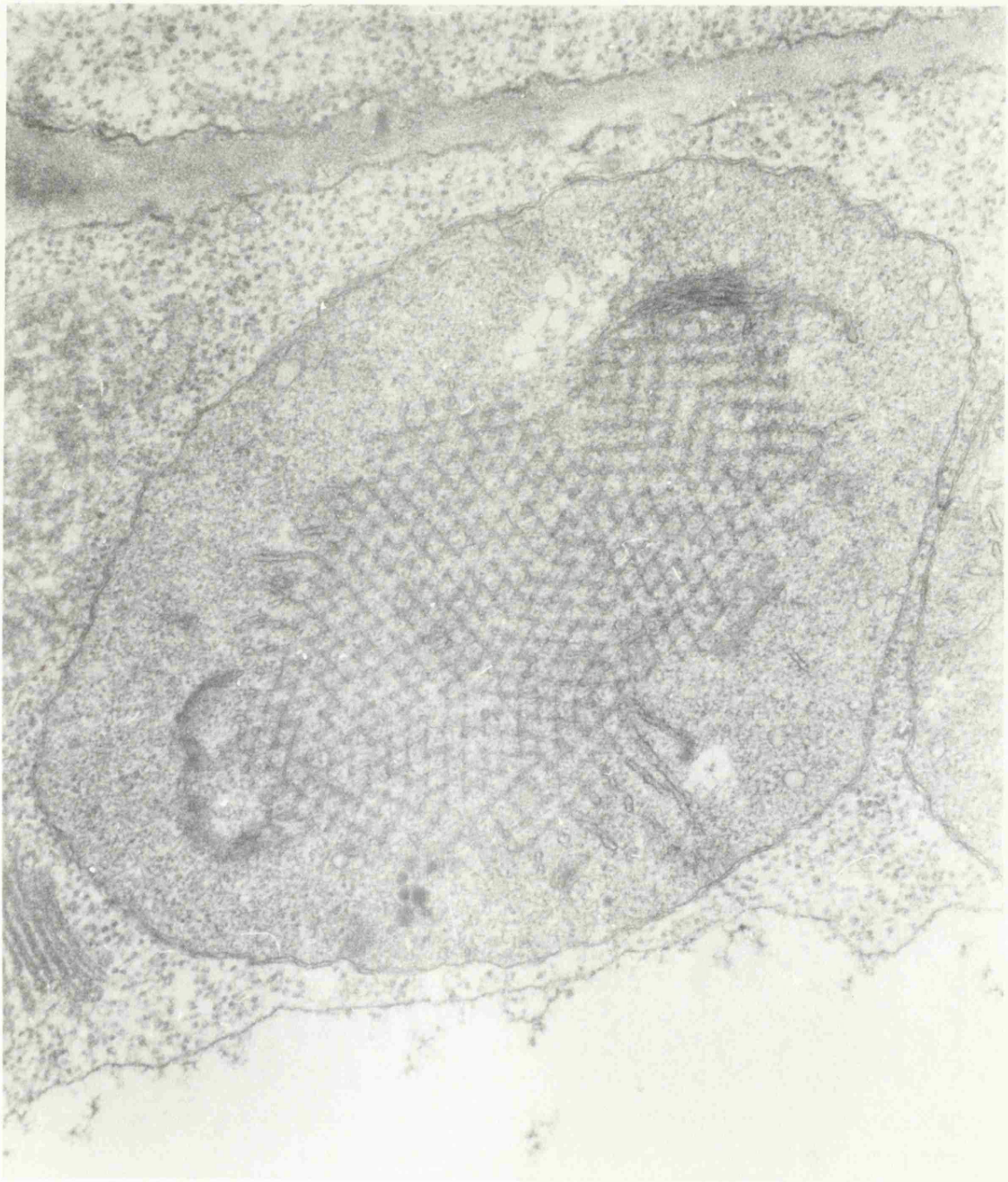


Plate 9: Oh CMU Magnification x 51,330

Plate 10: 3h CMU Magnification x 51,330

Plate 11: 6h CMU Magnification x 51,330

Plate 12: 9h CMU Magnification x 51,330

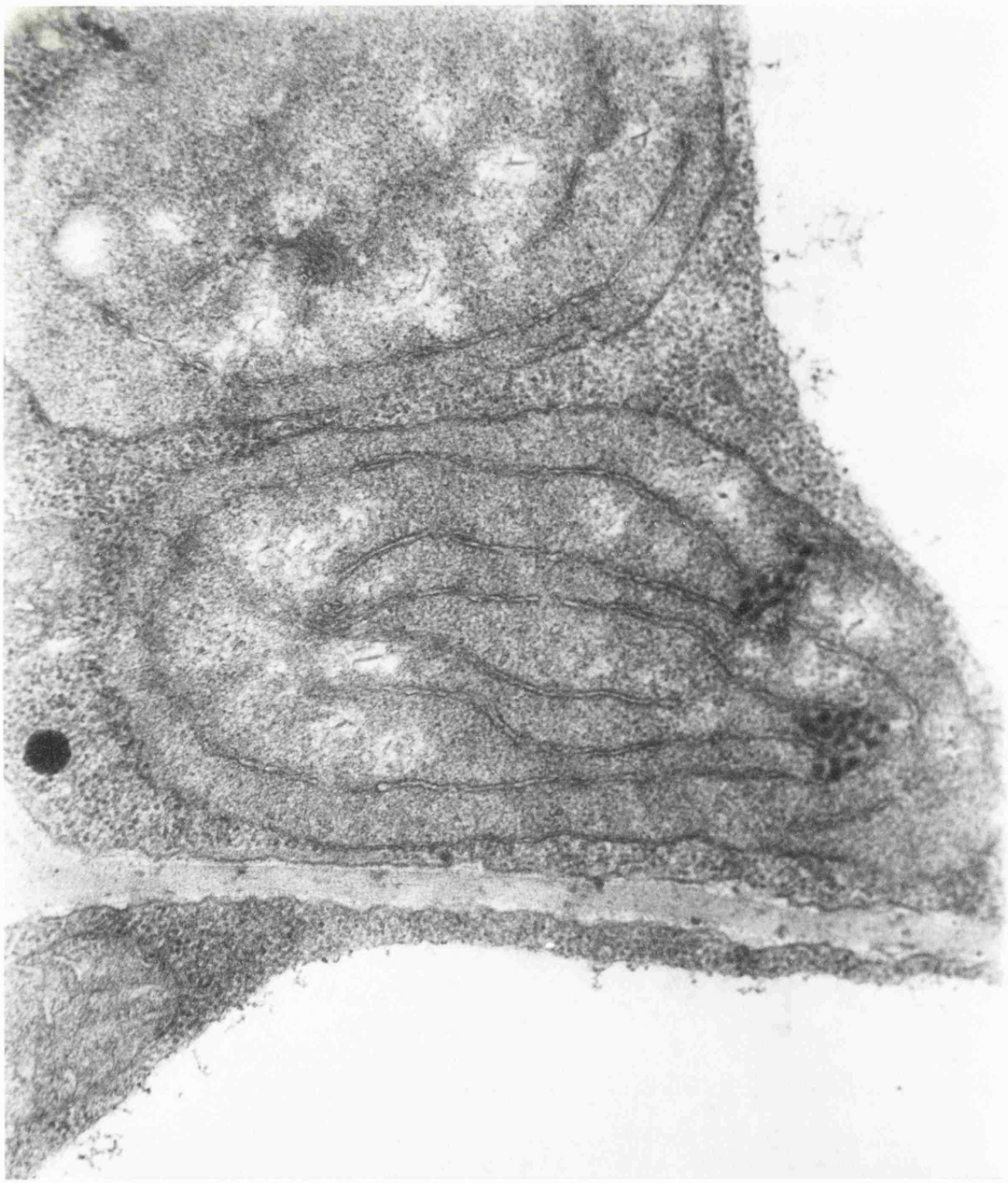


Plate 13: 12h CMU Magnification x 51,330

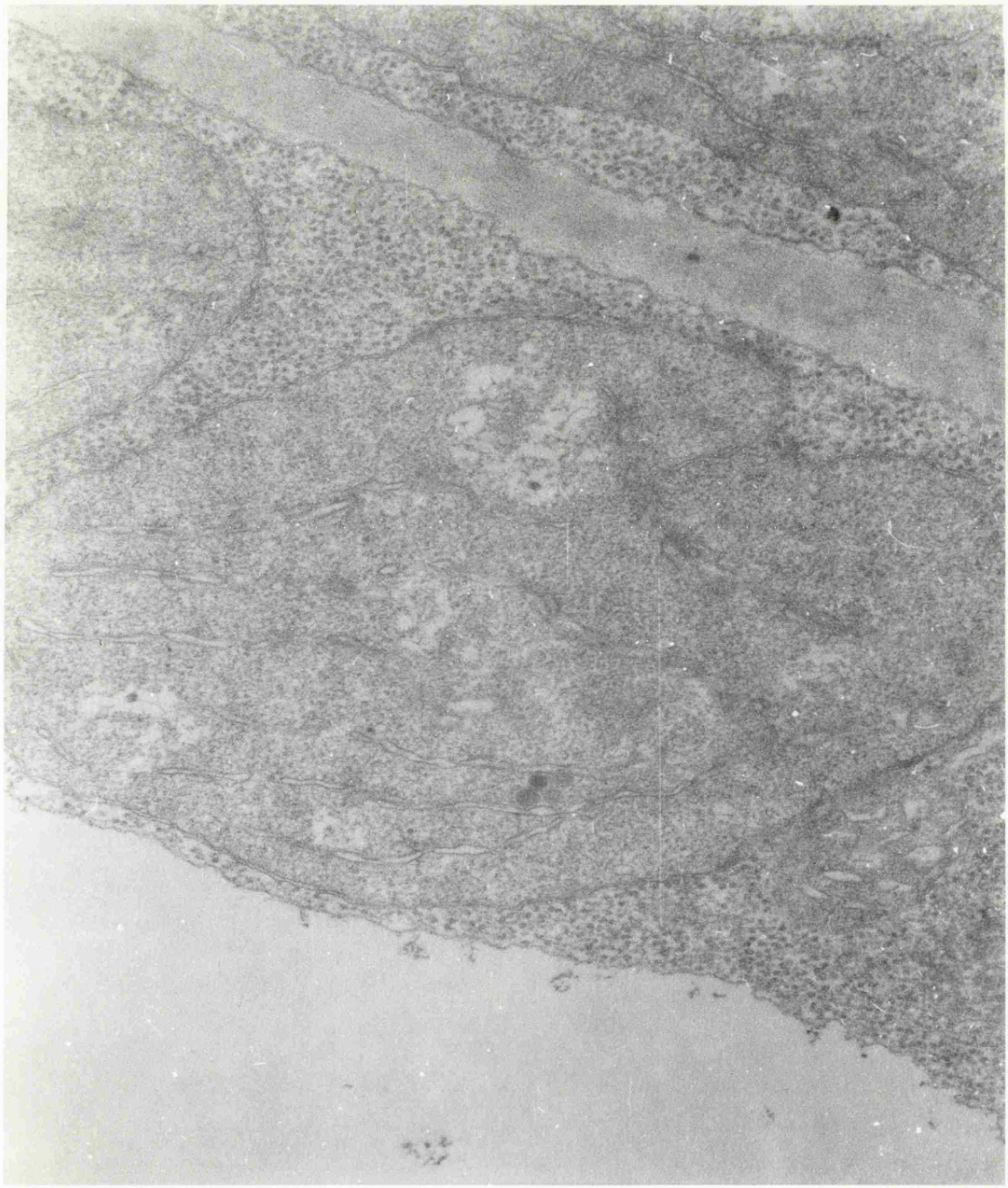


Plate 14: 24h CMU Magnification x 51,330

Plate 15: 30h CMU Magnification x 51,330

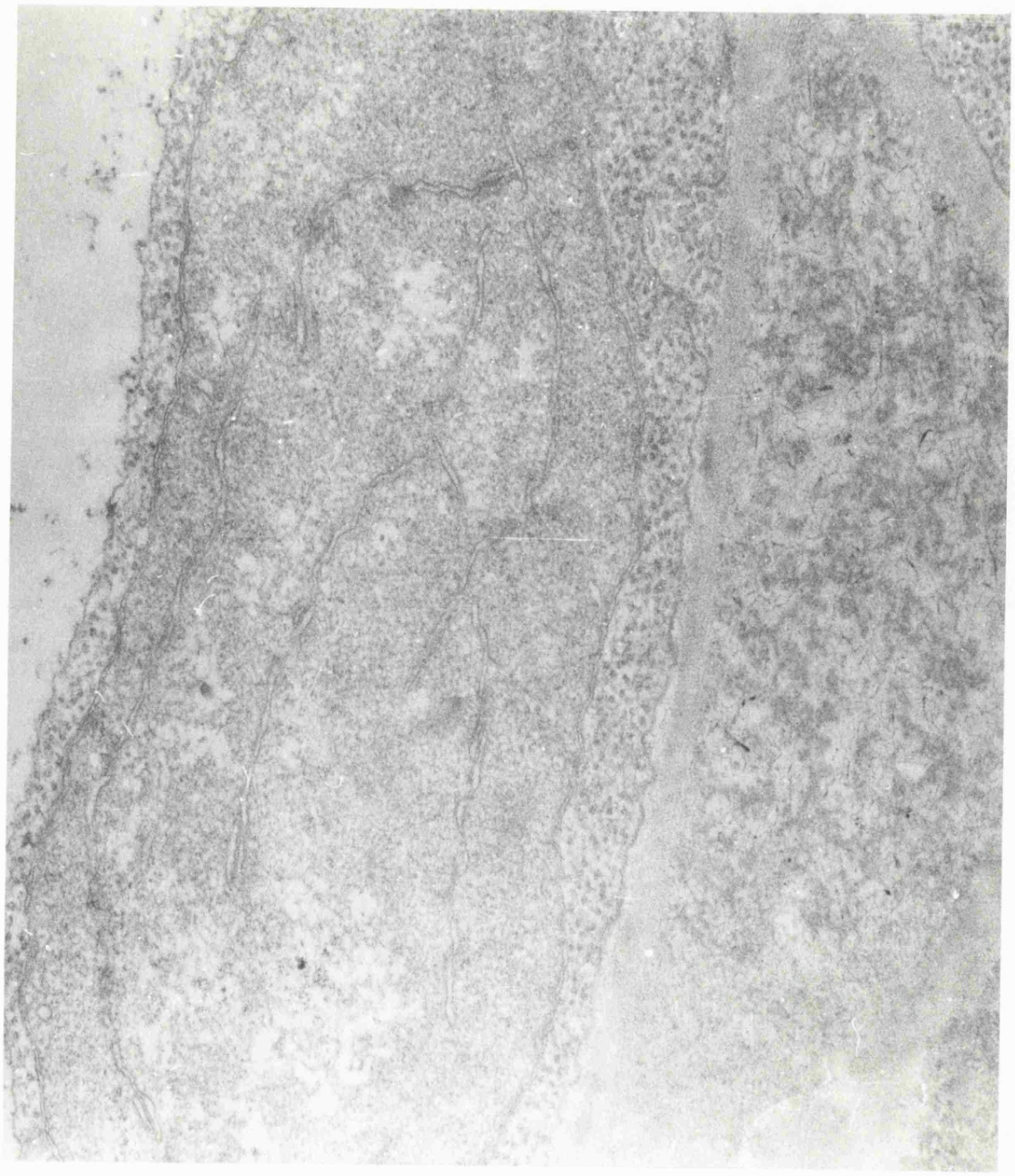


Plate 16: 48h CMU Magnification x 51,330

Plates 17 - 25: Changes occurring in the fine structure of chloroplasts in leaves treated with CMU/sucrose during 48h illumination. Leaf sections were pre-fixed with glutaraldehyde, post-fixed with osmium tetroxide and embedded in standard spur.

Plate 17: Oh CMU/sucrose Magnification x 51,330

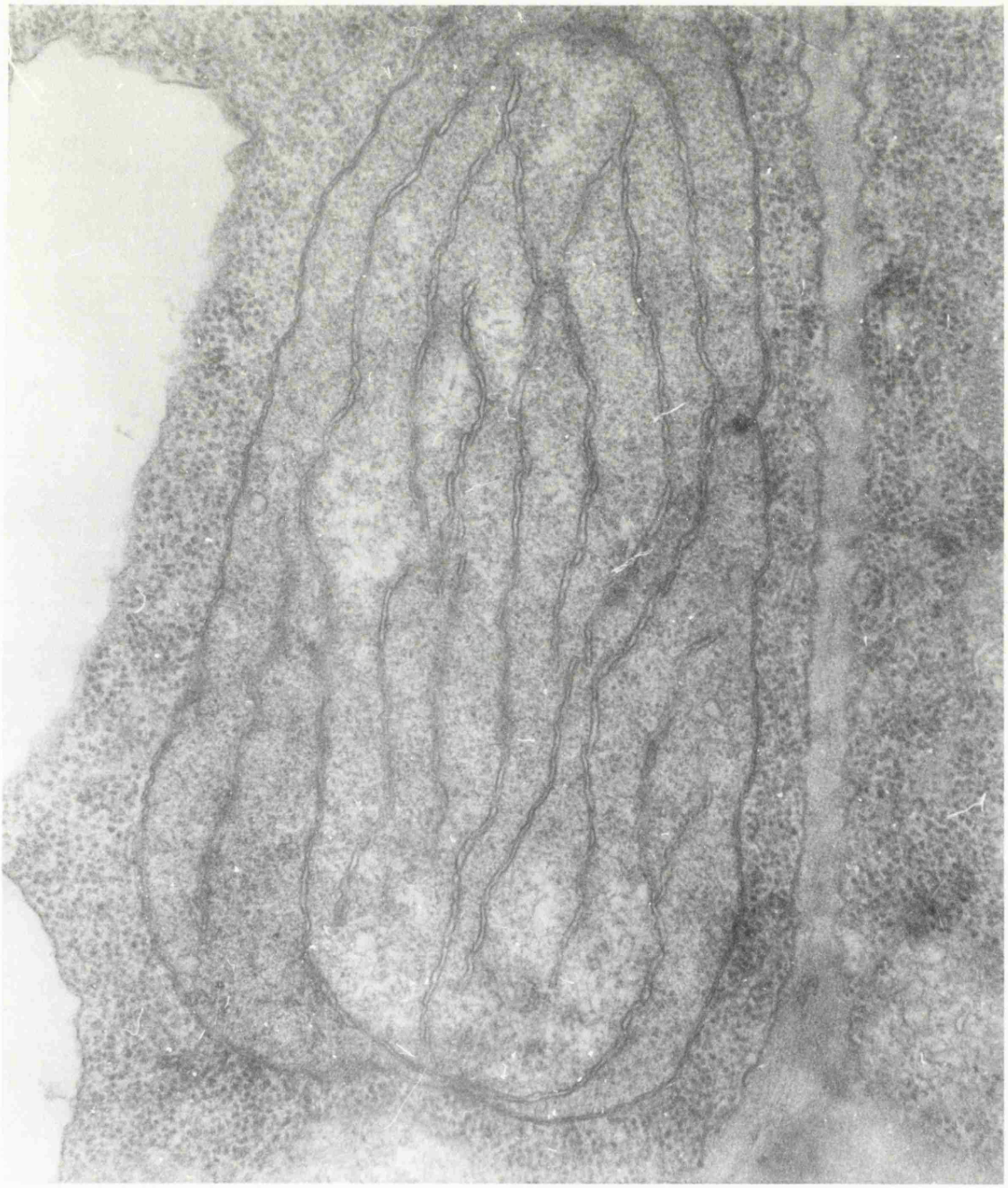


Plate 18: 3h CMU/sucrose Magnification x 51,330

Plate 19: 6h CMU/sucrose Magnification x 54,950

Plate 20: 9h CMU/sucrose Magnification x 51,330

Plate 21: 9h CMU/sucrose Magnification x 51,330

Plate 22: 12h CMU/sucrose Magnification x 51,330

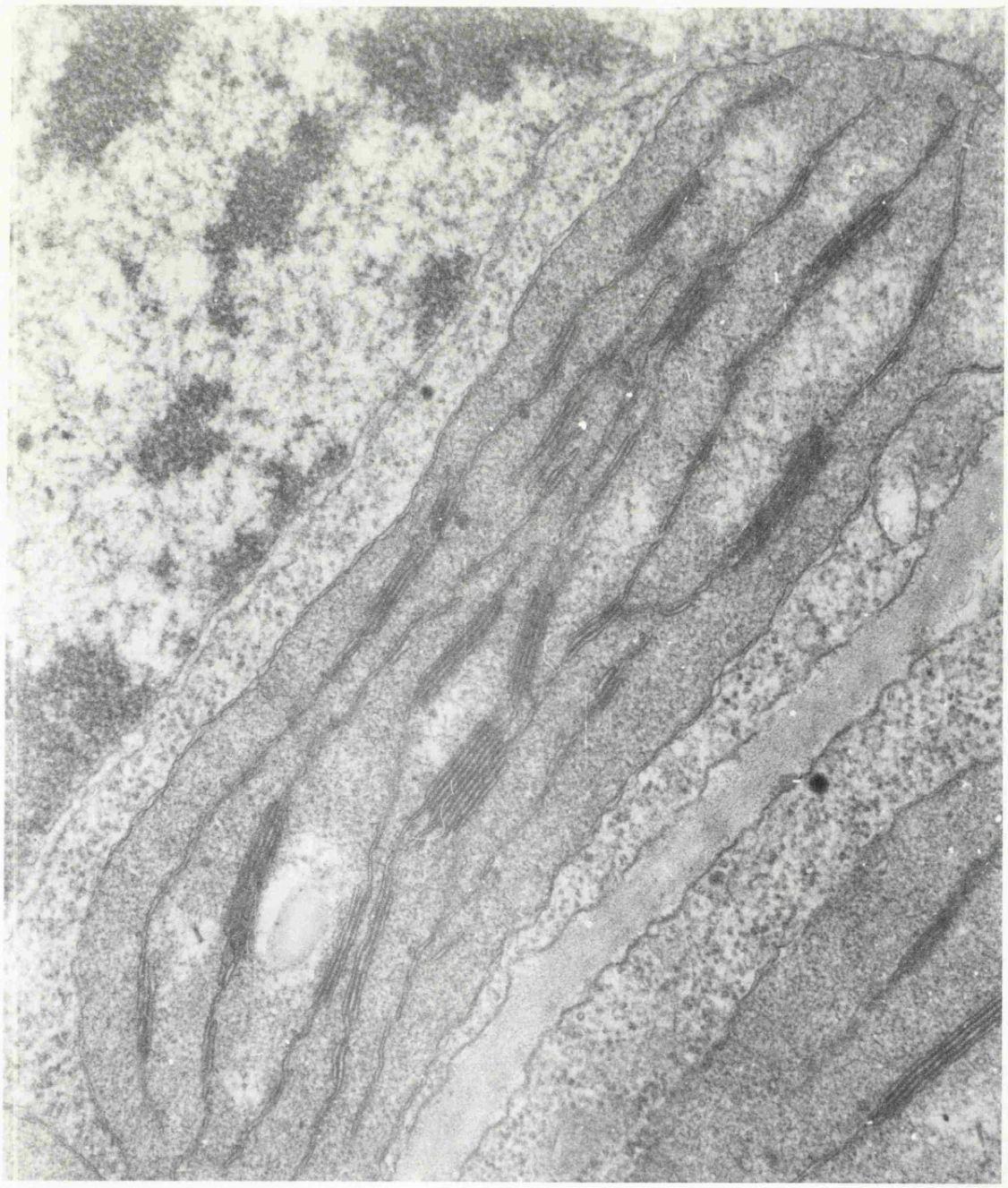


Plate 23: 24h CMU/sucrose Magnification x 51,330

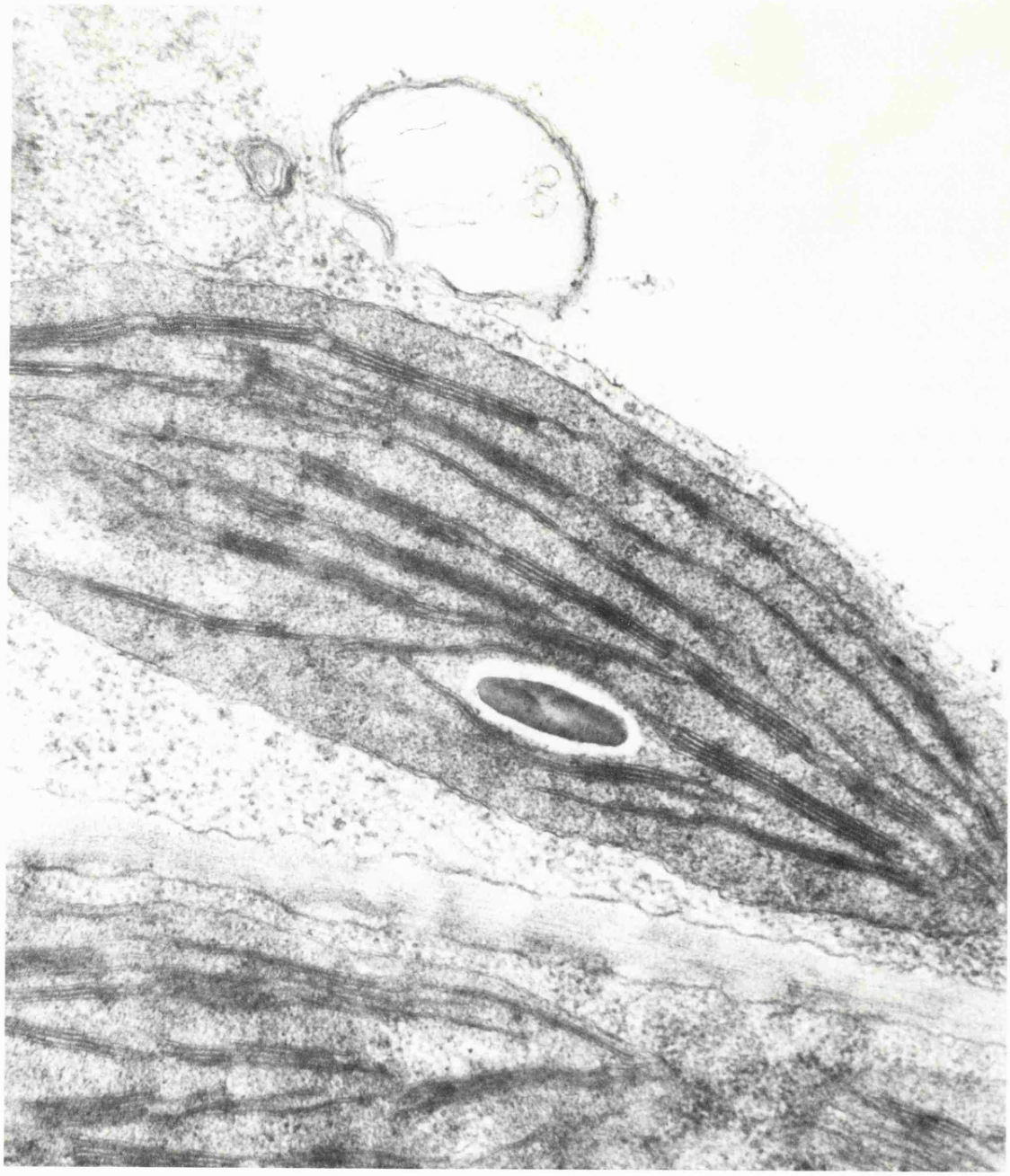


Plate 24: 30h CMU/sucrose Magnification x 51,330

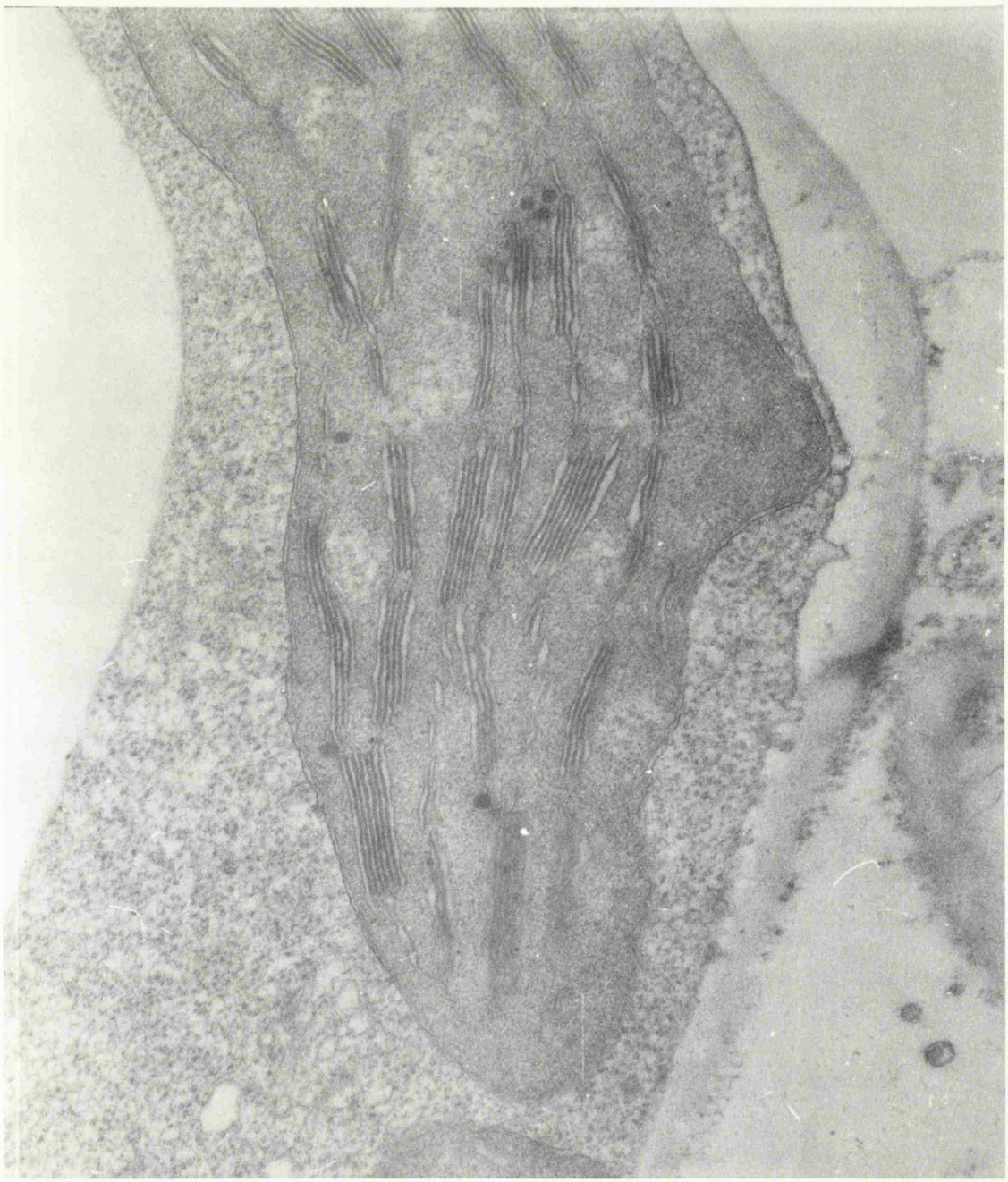


Plate 25: 48h CMU/sucrose Magnification x 51,330

Plates 26 - 33: Changes occurring in the fine structure of chloroplasts in leaves treated with water/sucrose during 48h illumination. Leaf sections were pre-fixed in glutaraldehyde, post-fixed in osmium tetroxide and embedded in standard spur.

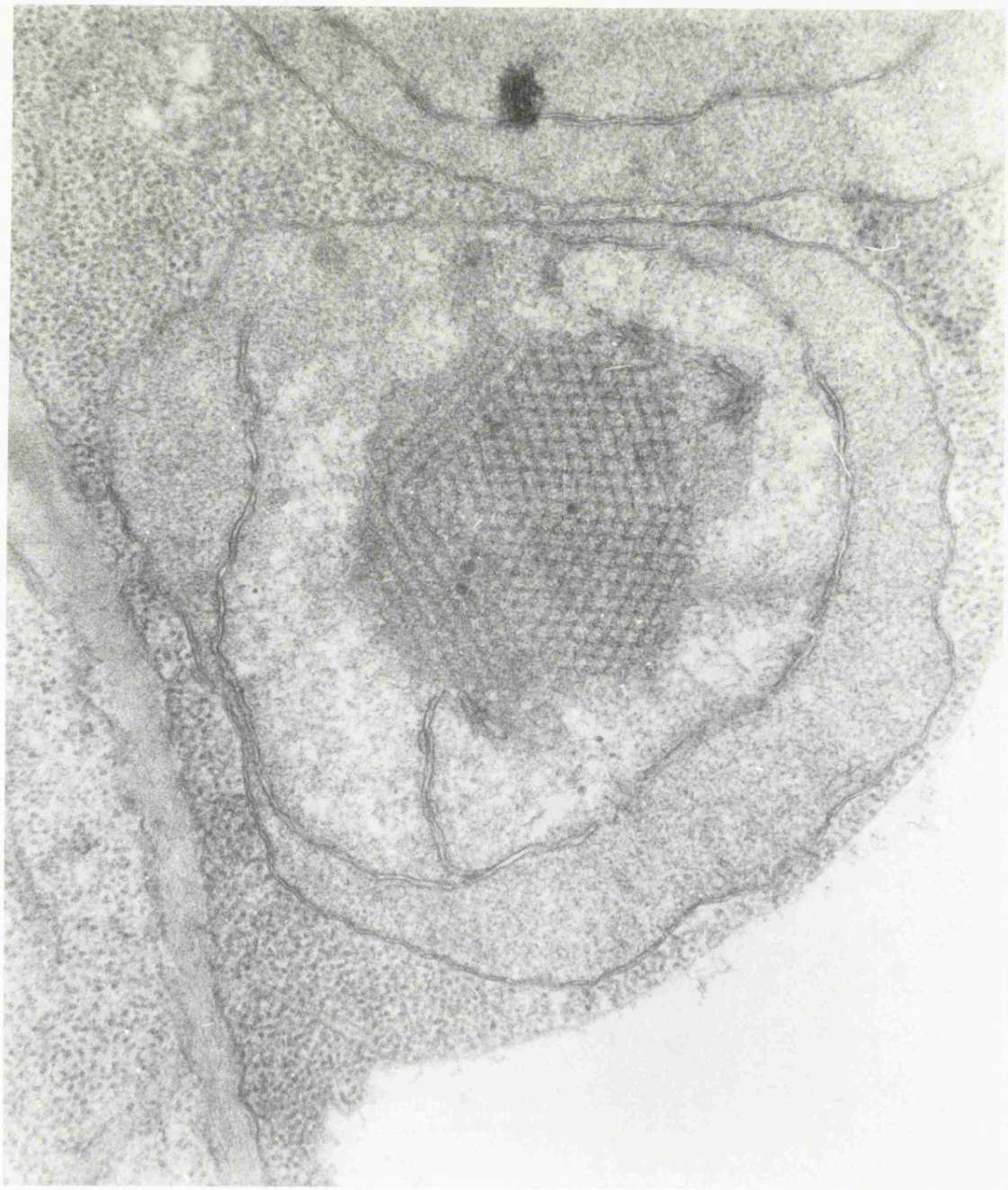


Plate 26: 0h water/sucrose Magnification x 51,330

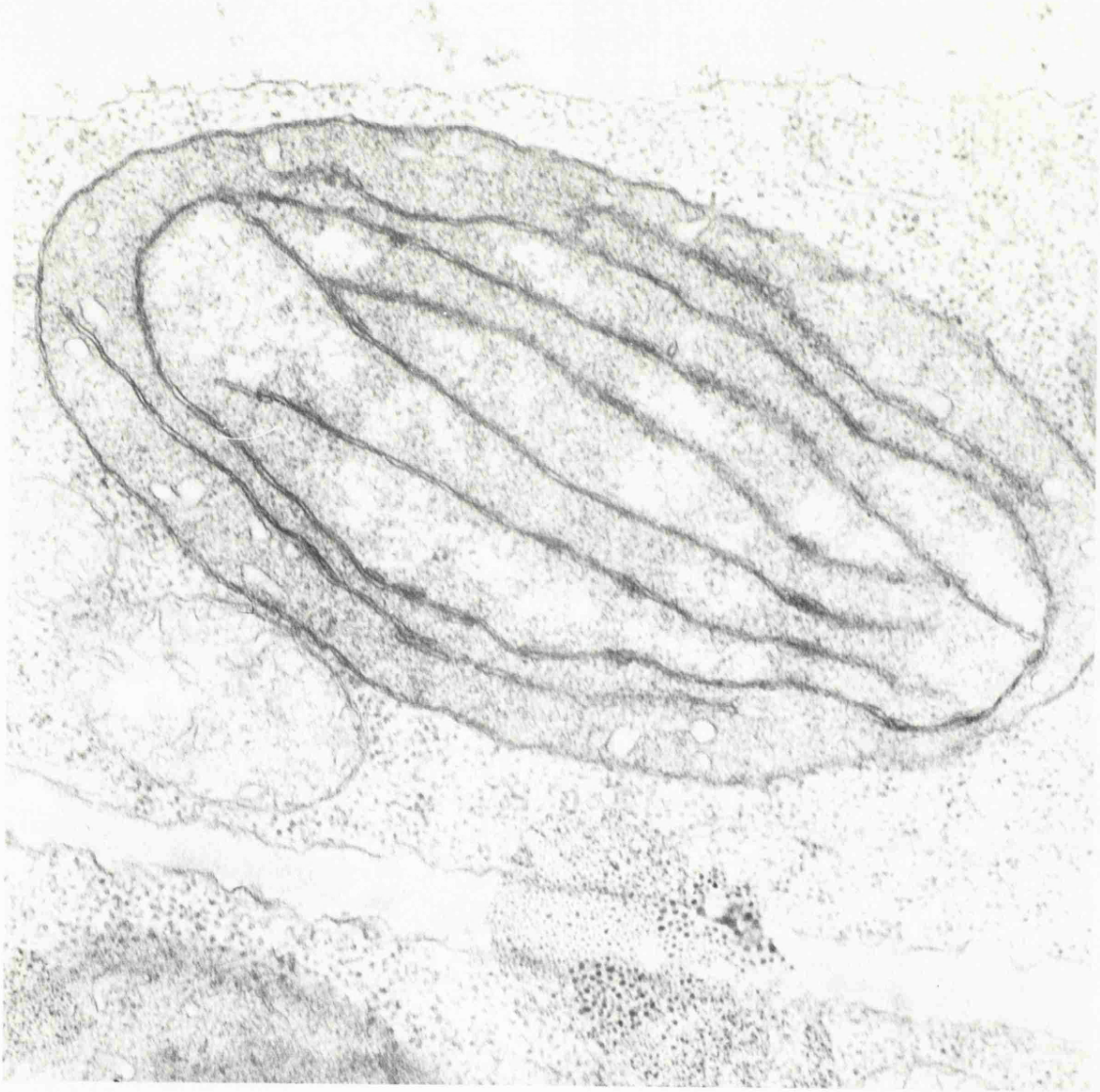


Plate 27: 3h water/sucrose Magnification x 54,950

Plate 28: 6h water/sucrose Magnification x 54,950

Plate 29: 9h water/sucrose Magnification x 54,950

Plate 30: 12h water/sucrose Magnification x 51,330

Plate 31: 24h water/sucrose Magnification x 51,330

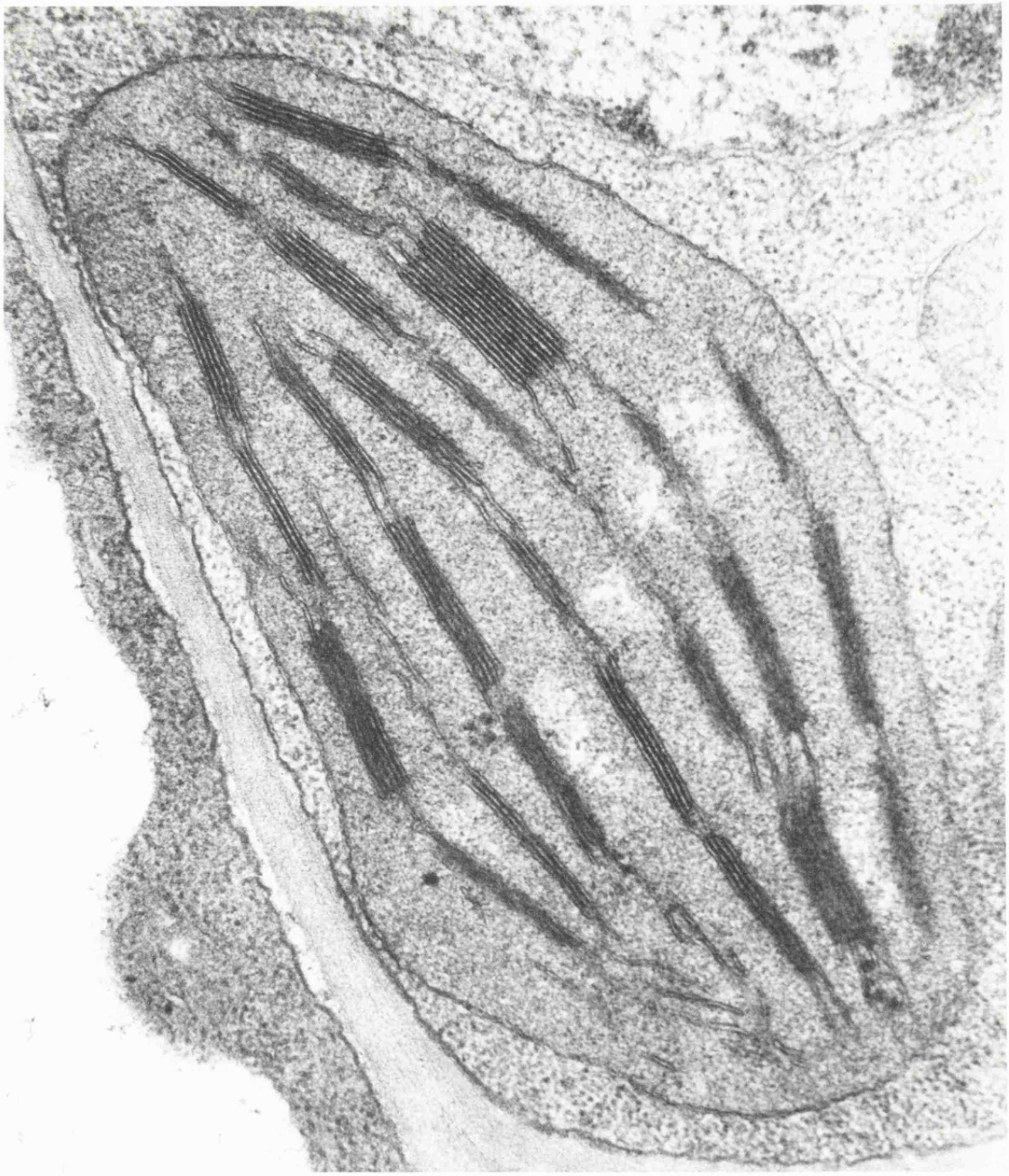


Plate 32: 30h water/sucrose Magnification x 51,330

Plate 33: 48h water/sucrose Magnification x 51,330

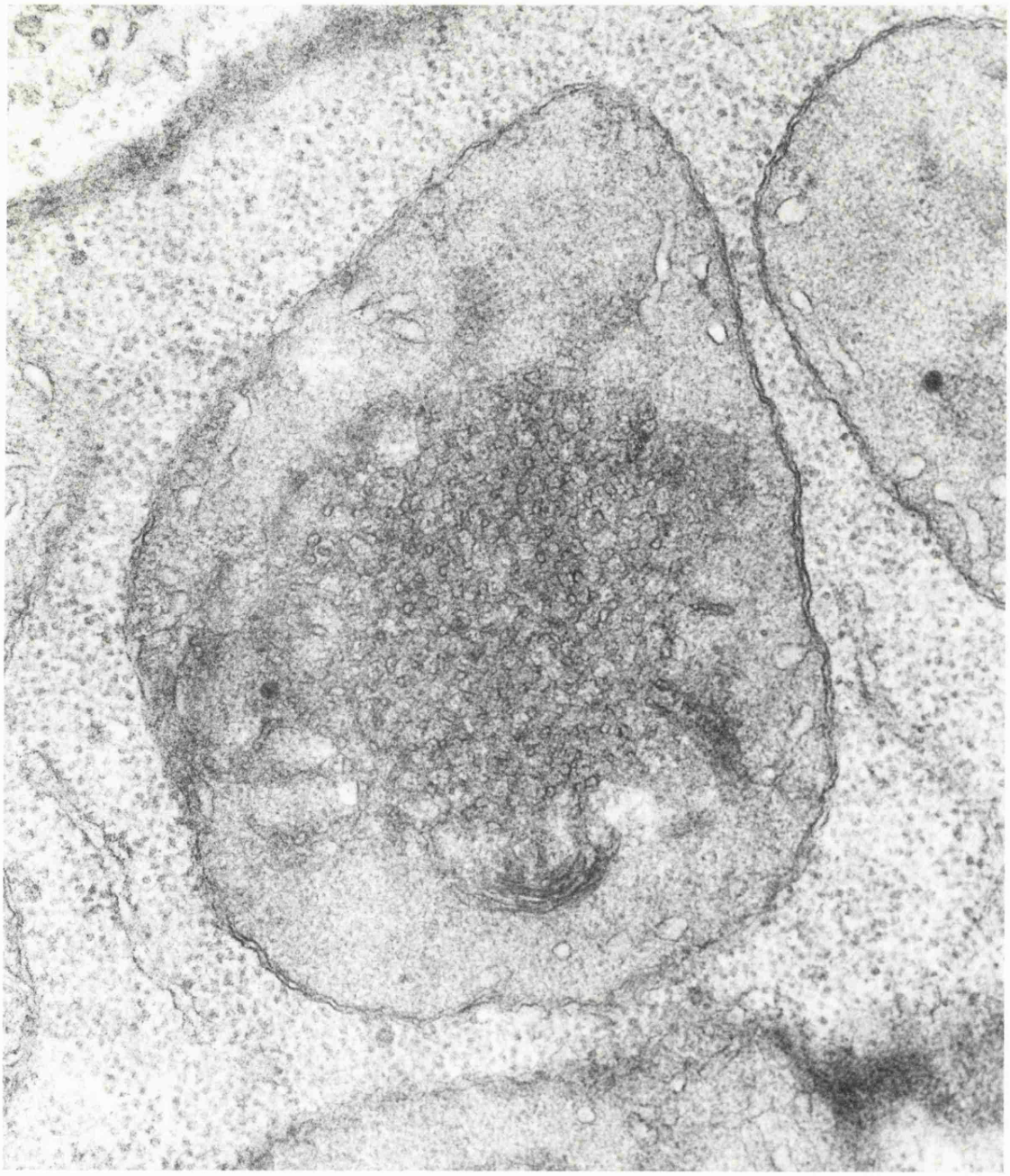


Plate 34: 10 min water control Magnification x 71,400

DISCUSSION

DISCUSSION

Extensive investigations have shown that when etiolated plants are illuminated the etioplast undergoes major structural changes as the conversion to a photosynthetically competent organelle takes place (see Introduction section 1.2). The problems of comparing data from different species and treatment conditions has already been outlined (Introduction section 6) and in this study care has been taken to standardise experimental conditions to allow a valid comparison of the development of photosynthetically functional and photosynthetically non-functional chloroplasts. In the first section of the discussion attempts will be made to correlate structure and function in developing chloroplasts. In the second section the role of photosynthesis in chloroplast development will be outlined. In addition, aspects of the functional integration and instigation of the photosynthetic electron transport will be discussed. In the third section evidence for the existence of cyclic phosphorylation *in vivo* and in its possible role in chloroplast development will be reviewed. In the final section some aspects of excitation energy transfer between the photosystems will be outlined.

1. THE CORRELATION BETWEEN CHLOROPLAST STRUCTURE AND FUNCTION

On illumination of etiolated pea explants there was an initial loss in the crystallinity of the prolamellar body, although the continuity of the membrane surface appeared to be maintained (plate 34). This change has been shown to coincide with the photoreduction of protochlorophyllide to chlorophyll (Klein et al., 1964). The relationship between these two light-induced events appears to be partly coincidental, since under certain experimental conditions the two processes can be made to occur independently. Virgin et al. (1963) found that 61% of the protochlorophyllide could be photoconverted within the prolamellar body without any change in the fine structure. More recently Treffrey (1973) showed that prolamellar bodies could be reformed under low light conditions without changes in the protochlorophyllide content. It is possible therefore that both processes are required for subsequent membrane biosynthesis but their interrelationship has yet to be ascertained.

Further illumination has been shown to result in a cleavage of the tubular connections of the prolamellar body and the dispersal of 'perforated' double membrane sheets or lamellae into the stroma (see Gunning and Steer, 1975). The rate at which the dispersal process takes place has been shown to be proportional to light intensity, and with sufficient intensity, can be complete within minutes (Virgin et al., 1963). In the present study, at a light intensity of 4 W/m^2, the process was complete within 3h. The dispersal of the prolamellar body was unaffected by CMU (plate 10) or by the absence of substrate. It is therefore concluded that the process is not energy requiring and

does not incorporate the synthesis of new material. This would confirm the theory that prolamellar body dispersal is a rearrangement of existing lamellae rather than the synthesis of new membrane (Gunning and Jagoe, 1967; Henningsen and Boynton, 1969; Bradbeer et al., 1973).

Occurring concomitantly with prolamellar body dispersal in this study, was the lag phase in chlorophyll synthesis. The two events are not dependent upon one another and increasing light intensity will, as already outlined, complete prolamellar body dispersal without effectively altering the chlorophyll lag phase. Conversely the latter was increased in explants by the removal of endogenous substrate or shortened by the addition of exogenous sucrose (Wolfe and Price, 1957 and figure 14) without any change in the rate of prolamellar body dispersal. It can be assumed therefore that the chlorophyll lag phase and associated chloroplast development is partially, although not completely, a feature of substrate shortage.

In addition to the lag phase in chlorophyll synthesis which is particularly evident, simultaneous lag phases have also been observed in cell growth, and in the activities of some chloroplast enzymes (Bradbeer, 1970). Furthermore this study has shown a lag phase for carotenoid synthesis (figures 16 and 17), manganese incorporation (figure 22) and in the formation of proteins associated with the pigment-protein complexes (figures 28 and 29). The length of the lag phase of these components was similar to that of chlorophyll and could also be shortened by the exogenous application of sucrose. It is speculated that although little or no synthesis of membrane components could be observed, the lag phase is a period of intense biochemical

activity during which components, stored in the etioplast, are inserted into the dispersing lamellae and organised in such a way as to facilitate a functional PS I. Although the synthesis of membrane components is increased by addition of substrates there is no present evidence to suggest that the assembly of these components into the membrane, and hence the onset of PS I activity, is shortened.

Between 3 and 4h after the onset of illumination PS I activity as measured by ascorbate photooxidation (figure 23) and the accumulation of low levels of chlorophyll (figure 14) and β-carotene (figure 16) were detected. Approximately concomitant with, and possibly correlated to these increases in activities, was the disappearance of the perforated membrane sheets and the formation of featureless primary thylakoids. This observation confirms those of Sironval et al. (1968b)and Phung-Nhu-Hung et al. (1970 a and b) who showed the presence of an active PS I together with primary thylakoids in chloroplasts isolated from flash illuminated leaves. It is possible that primary thylakoid formation would require a small amount of membrane biosynthesis, which could be reflected in the synthesis of the basic membrane building block for PS I, chlorophyll-protein complex I. However, the results of this study (figure 29) correlate with the experiments of Alberte et al. (1972) who showed that this protein was not synthesised in greening Jack Beans until after approximately 5h illumination (see Discussion section 2). The synthesis of low levels of chlorophyll-protein complex II was however detected (figure 28). The recent observation that this complex is a source of antennae chlorophyll for both PS II and PS I (Thornber, 1975) would infer that

primary thylakoid formation and the small increases of chlorophyll are due to the synthesis and incorporation of this chlorophyll-protein complex into the developing membrane. The small amounts of chlorophyll-protein complex I present in the etioplast are all that is required for initial PS I activity but it is complex II which is synthesised for the purpose of providing excitation energy to PS I at this stage.

The synthesis of chlorophyll-protein complex II and β-carotene in plants devoid of endogenous substrate and prior to the onset of functional PS II would suggest that concomitant with PS I induction, an energy producing mechanism is also organised. This could be cyclic phosphorylation which has been shown to commence at a similar point in time (Dodge and Whittingham, 1966; Gyldenholm and Whatley, 1968; see also Discussion section 3). In this present study cyclic phosphorylation remained undetected until after approximately 6h illumination but the initial levels were particularly high (figure 25). Due to the number of parameters involved in calculating the levels of cyclic phosporylation, it is possible that the assay is insufficiently sensitive when compared with ascorbate photooxidation. If this is so, the curve for cyclic phosphorylation could be extrapolated back to a time similar to the onset of PS I activity.

Between 5 and 6h illumination the induction of PS II was detected by both ferricyanide and silicomolybdate reduction (figures 20 and 21). Corroborative evidence for PS II induction at this time was shown by the detection of whole chain electron transport (figure 19) and photosynthetic CO_2 uptake in whole leaves (figure 18). The major structural change which occurs simultaneously to the onset of

PS II activity is the formation of grana stacks, by protrusion and helical overlapping of newly synthesised lamellae from the original primary thylakoids (see Introcuction section 1.3). The synthesis of new membrane is reflected in the simultaneous synthesis of the two chlorophyll-protein complexes as resolved by SDS polyacrylamide electrophoresis of solubilised chloroplast lamellae. Associated with the chlorophyll-protein complex and synthesised also were larger amounts of chlorophyll, β-carotene, lutein, and an increase in PS I activity.

The physiological role of grana stacking has stimulated considerable speculation for many years. It can be concluded from the results presented in this study, in addition to those previously reviewed (see Introduction section 5.2) that PS I and possibly cyclic phosphorylation do not require grana stacking. In addition high rates of these activities have been shown in fractionated stroma lamellae (Sane et al., 1970), C_4 agranal chloroplasts (Smillie et al., 1971) and mutants containing no stacked lamellae (Goodenough et al., 1969).

There is considerable published data which strongly supports the concept that PS II activity is dependent upon grana formation. Initial studies on the agranal bundle sheath chloroplasts of C_4 plants using TNBT chloride as a Hill electron acceptor (Downton et al., 1970) suggested that PS II activity was absent. These results were confirmed by Woo et al. (1970) using $NADP^+$ as the terminal electron acceptor. Subsequent experiments by Bishop et al. (1971) and Smillie et al. (1971) showed PS II mediated ferricyanide and DCIP reduction to be present in bundle sheath chloroplasts. These results suggested that the absence

of PS II activity, shown by the whole chain assays of Woo et al., was due to a block in electron transport after PS II. Addition of plastocyanin (Smillie et al., 1971) effected a resumption in whole chain electron transport in these chloroplasts. The rates of PS II activity obtained in these agranal bundle sheath chloroplasts was only 7 - 14% of those obtained from the grana-containing mesophyll cells, but no direct correlation could be made between the level of PS II and extent of grana stacking.

Studies using chloroplast mutants have also provided conflicting evidence on the role of grana stacking. Perhaps the most convincing correlation between PS II activity and stacking has been provided by the experiments of Homann and Schmid (1967) using a yellow leaved tobacco mutant. The chloroplasts lacked both grana stacking and PS II activity. Equally convincing but contrasting results were published by Goodenough and Staehelin (1971), who showed that agranal chloroplasts from two Chlamydomonas reinhardii mutants produced levels of PS II activity above those of the controls. In addition, studies with pea (Highkin et al., 1969) and soybean (Keck et al., 1970) mutants, showed no correlation between a limitation in chloroplast membrane structural organisation and photochemical activity.

Izawa and Good (1966 a and b) have shown that the grana of higher plant chloroplasts can be dispersed by low salt washing without any loss in PS II activity. Despite this and other compelling evidence which demonstrated that PS II did not require stacked grana, it is also very clear from the membrane fractionation studies of Park and Sane (1971) that PS II is localised in the grana regions of mature

spinach chloroplasts. In this present investigation it has been demonstrated that after 30h greening of CMU/sucrose and H_2O/sucrose treated explants (plates 24 and 31) there was a structural disorganisation of the grana, which correlated with a considerable loss in PS II activity (figure 20).

The evidence reviewed above suggests, although not conclusively, that PS II can function without the presence of grana stacking, and that any correlation between the two is only apparent. However, there is a possibility that a reverse relationship might exist between the two processes, in that one or more of the components associated with PS II might be required to produce a molecular environment conducive to membrane appression. Closely associated with the building blocks of the photosynthetic membrane is the tetrapyrrole moiety of chlorophyll. Chlorophyll b in particular has been associated with PS II (Park and Sane, 1971) and it is noticeable that levels of this pigment increase prior to the onset of PS II (Anderson and Boardman, 1964). In addition, the chlorophyll a:b ratio has also been closely correlated with stacking in the bundle sheath and mesophyll chloroplasts of C_4 plants. A decrease in the chlorophyll b content is reflected by reduced stacking (Pyliotis et al., 1972). Conversely Goodchild et al. (1966) have shown the presence of grana stacks in a chlorophyll b deficient barley mutant, which suggests that other components independently of, or in addition to, chlorophyll b, are responsible for stacking.

Anderson (1975) in a recent review has suggested that the chlorophyll-protein complex II might be responsible for grana stacking,

since mechanical fractionation procedures have detected its presence in stacked lamellae. Moreover original fractionation procedures using digitonin (Thornber et al., 1967) and Triton X-100(Remy, 1971) have shown the PS II fraction to be enriched with this major intrinsic protein. Doubt has been cast on the contribution of chloropyll-protein complex II to stacking since the agranal chlorophyll b deficient barley mutant is rich in this protein (Sagromski and Döbel, 1974). The time course for the synthesis of this complex during greening is shown in figure 28. Initial synthesis was demonstrated after 3h which would correlate well with PS I, rather than with PS II and stacking. In addition, substantial amounts of the protein were evident in the lamellae of chloroplasts isolated from CMU treated explants and which were unable to form grana stacks (plates 9 - 16).

Increases in lutein (figure 17) correlated well with grana formation, and since it is now established that xanthophylls are generally associated with PS II, it is envisaged that they might also play a role in stacking. Little information is presently available on xanthophylls but valuable experience could be gained from the use of a specific carotenoid inhibitor such as Sandoz 6706 (Bartels and Hyde, 1970). Manganese incorporation also occurred concomitantly with grana stacking (figure 22). Although it is well established that PS II activity is dependent upon manganese incorporation (Cheniae, 1970) much of this can be removed without loss of structural integrity (Homann, 1967). The structural manganese complement is shown in figure 22 by the amount present in etiolated membranes. A loss in this content correlated well with photodestruction of the membrane in the

CMU treatment. The functional manganese content was assumed to be that which was incorporated simultaneously with increasing oxygen evolution during greening.

Boardman et al. (1970) have shown that in chloroplasts isolated from greening pea leaves detectable levels of cytochrome b559 (high potential) appeared to coincide with grana formation and increasing variable fluorescence changes. PS II activity has been detected prior to this and therefore independently of the presence of grana or cytochrome b559 (Hiller and Boardman, 1971).

The evidence at present favours the concept that grana stacks are not required for a functional PS II. However, it might be speculated that there is a close relationship between the components of PS II and those required to produce tight stacking. There is no evidence available, however, to suggest which component or components are responsible but it would appear that stacking is probably a complex interaction between several membrane components which can be regulated by presence of salts (Izawa and Good, 1966 a and b).

In conclusion the biosynthesis of the photosynthetic membrane, in this and in other studies, appears to be a regulated multistep process (see figure 33). The light-induced dispersal of the prolamellar body allows the organisation of PS I components, which are present in small quantities in the etioplast, to occur during what has been referred to as a lag phase. The onset of PS I and possibly cyclic phosphorylation enables the simultaneous induction of PS II and membrane biosynthesis, by protrusion of new lamellae from the existing primary thylakoid. One or more of the components of PS II possibly

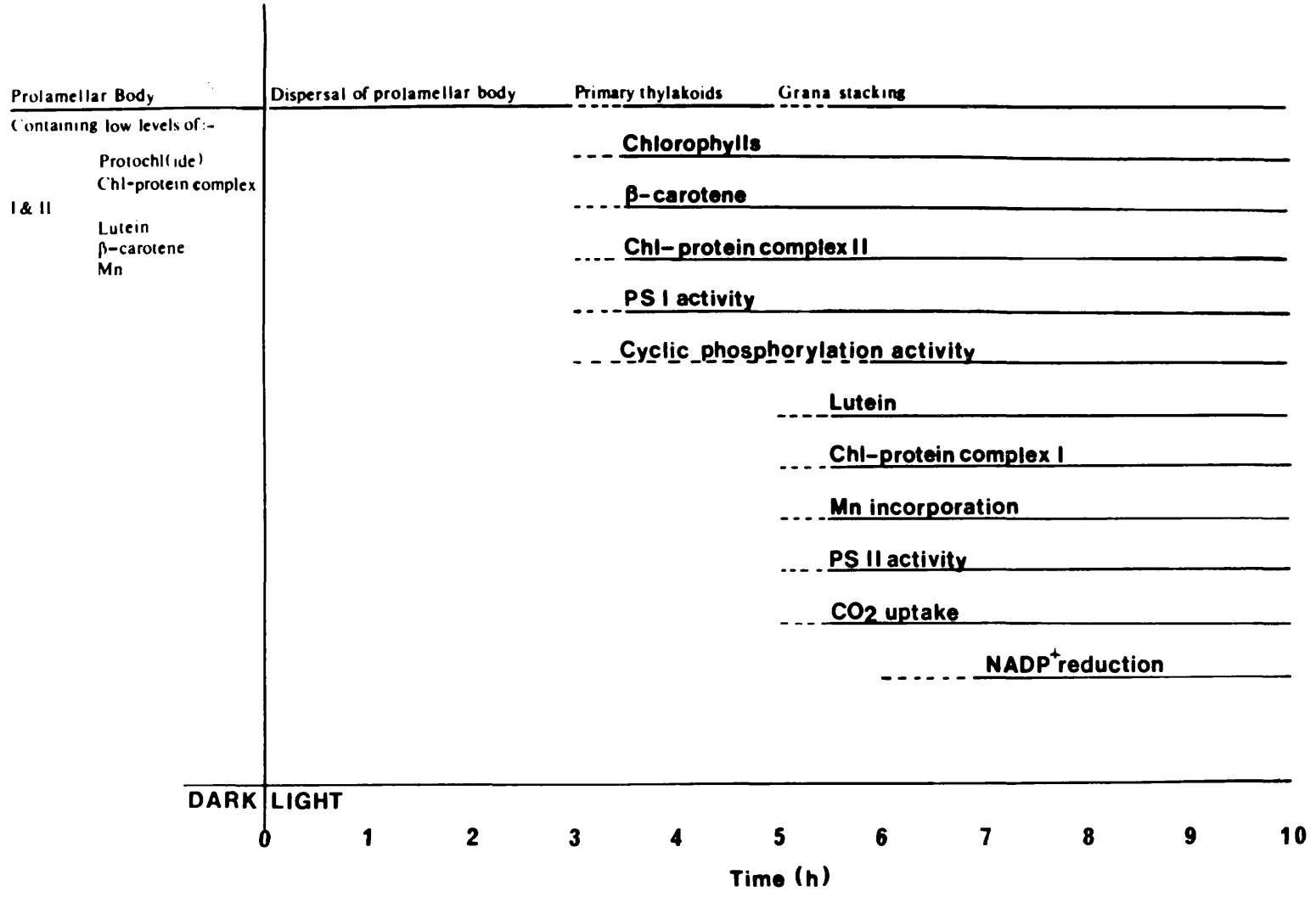


Figure 33. Diagram showing the appearance of structural and biochemical events occurring in dark grown chloroplasts from control pea explants over a period of 10h illumination. The broken line represents the extrapolated time of appearance of each event and the continuous line shows the actual observed appearance.

provides the "glue" which is required for the molecular bonding of the grana partition and therefore PS II is confined to this electron dense area of the grana (Park and Sane, 1971). Cleavage of these bonds by the addition of salts (Izawa and Good, 1966 a and b) can therefore result in the loss of stacking without a corresponding loss in PS II activity. PS I components are also incorporated into the new lamellae, and are reflected by increases in the level of the chlorophyll-protein complex I at the same time as grana formation. Also synthesised and shown by grana formation is the chlorophyll-protein complex II and although not associated directly with PS I or PS II function it is the major light harvesting protein in the photosynthetic membrane. Its high complement of chlorophylls a and b allows efficient absorption of light which can be distributed to the reaction centres of PS II and PS I as required.

The final step in photosynthetic membrane biosynthesis appears to occur between 6 and 9h after the onset of illumination. This is the synthesis of ferredoxin-$NADP^+$reductase (figure 24) and this is consistent with the results of Gyldenholm and Whatley, (1968) and Bradbeer et al., (1970).

2. THE CONTRIBUTION OF PHOTOSYNTHESIS TO CHLOROPLAST DEVELOPMENT

The energy requirement for the growth and maintenance of a mature plant is completely provided for by photosynthesis and can thus be described as being autotrophic. During the growth of a seedling

and prior to establishment of photosynthetic competence, the plant is dependent upon its cotyledon(s) for a supply of substrate. Some part of the stored substrate will provide for chloroplast development, at least until photosynthesis can produce an alternative source. A major part of this study has been to establish exactly how much photosynthesis is required for total chloroplast development and whether it can be substituted by an external carbohydrate supply. Previous studies (see Introduction section 6) have shown that the development of chloroplast fine structure and chlorophyll synthesis in photosynthetically incompetent higher plants and algae, is unaffected, provided an exogenous sucrose supply is present.

Although endogenous substrate provided by the cotyledons is undoubtedly utilised in chloroplast development, figure 18 shows that photosynthetic competence can be achieved in explants devoid of substrate. Photosynthesis in this study, was measured by CO_2 uptake using infra red gas analysis techniques. The measurements may therefore be representative of 'apparent' rather than absolute photosynthesis since it must be acknowledged that actual CO_2 uptake by the leaves could be offset by light-induced respiration. Nevertheless the exogenous addition of sucrose to greening explants produced detectable rates of CO_2 uptake prior to those in the substrate deficient water controls. Therefore, although substrate was not required for the onset of photosynthesis the preceeding lag phase leading to photosynthetic competence was considerably shortened. The exogenous application of sucrose has also been shown to shorten the lag phase in chlorophyll synthesis (Wolfe and Price, 1957; Sisler and Klein, 1963; see also

Discussion section 1).

Chlorophyll synthesis in plants is an energy requiring process and the rapid accumulation which occurs during chloroplast development is normally associated with the induction of PS II and consequent onset of photosynthesis. Inhibition of photosynthesis by CMU resulted in the total inhibition of further chlorophyll synthesis after the initial photoconversion of endogenous precursor. Klein and Neuman (1962) published similar results from their experiments with greening bean leaves in which chlorophyll synthesis during the first 24h of illumination was only inhibited by 50% but this increased to 100% during further illumination. The discrepancy between their results and those of this study may be explained by failure on their part to remove endogenous substrate prior to illumination. Inhibition of chlorophyll synthesis by CMU was reversed to levels above those of the water control by the exogenous application of sucrose. It is concluded that the role of photosynthesis with respect to chlorophyll synthesis is only one of supplying carbohydrates and can be easily substituted.

Small increases in chlorophyll content were observed in the leaves of the water control and water/sucrose treatments between 3 and 4h after the onset of illumination and approximately 2h prior to the detection of photosynthesis. Furthermore as the water control was devoid of other substrate for chlorophyll synthesis, it would appear that a further source of energy was present at this point. The concomitant induction of PS I activity with these increases in chlorophyll levels, might suggest the involvement of cyclic phosphoryl-

ation (see Discussion section 3). The induction of PS I prior to photosynthesis and also independently of the presence of substrates would suggest that similar chloroplast development might be expected in the chloroplasts of the CMU treated plants. This however was not observed and may be explained by the fact that either PS I induction or cyclic phosphorylation was inhibited by CMU. Figure 23 shows that the onset of PS I in CMU/sucrose treated explants was not detected until approximately 3h later than in the water controls, and thus cyclic phosphorylation would also not be present. CMU inhibition of PS I cannot be explained.

The chlorophyll-protein complex I is closely associated with, and generally thought to be the structural representation of, the PS I reaction centre. Thus, it might be expected that the onset of PS 1 activities in this study, should be accompanied by a synthesis of this complex. This was not the case, however, and it would appear that the insertion of the small levels present in the etioplast (figure 29), into the developing membrane, is all that is required for the induction of PS I. The presence of this complex was detected in each of the four treatments and therefore cannot be the factor limiting PS I induction in the CMU and CMU/sucrose treated explants. Present also in the internal membrane of the etioplast, and in addition to chlorophyll-protein complex I, were small levels of chlorophyll-protein complex II (figure 28). The absence of this complex in some photosynthetically active plants (Boardman and Highkin, 1966) together with the fact that it contains 40 - 60% of the total chloroplast chlorophyll, would suggest that it is the major light harvesting component for both

photosystems (Thornber and Highkin, 1974) but independent of functional activity. Further synthesis of chlorophyll-protein complex II in this study, coincided with the induction of PS I, and it is possible that the increase in chlorophyll which occurred with the onset of PS I activity can be attributed to synthesis and insertion of this light harvesting chlorophyll-protein complex into the membrane. Alberte et al. (1972), also observed the formation of this complex prior to that of chlorophyll-protein complex I in greening Jack Beans. At that time the protein was thought to be the structural manifestation of the PS II reaction centre and they consequently but wrongly concluded that PS II appeared in the photosynthetic membrane prior to PS I. The synthesis of chlorophyll-protein complex II also appears to occur in the absence of photosynthesis or a substrate supply, which confirms the operation of an additional energy producing mechanism. In addition, substantial increases in the content of the complex were detected in the chloroplasts of CMU treated plants which suggests that it is synthesised without the synthesis of its associated pigment and also without comcomitant organisation of the internal membrane.

Although it would appear that the insertion and organisation of PS I components does occur prior to photosynthetic competence, subsequent increases in the rates of PS I activity are concomitant with new membrane synthesis in the form of grana which are dependent upon the onset of photosynthesis. The rates of PS I in CMU/sucrose treated explants, however, after an initial lag, were similar to those of the water control, and this would again suggest that photosynthesis was non-specific. Contrasting results were obtained when PS I activity,

measured by $NADP^+$ reduction with ascorbate as electron donor, were compared (figure 24). After 24h the activity in chloroplasts isolated from the CMU/sucrose treated explants was 66% of the water control rate. Since the ferredoxin used in this assay was low in reductase activity it would appear that when PS II electron flow was blocked by CMU, ferredoxin-$NADP^+$ reductase synthesis was inhibited. The requirement for photosynthesis in the synthesis of ferredoxin-$NADP^+$ reductase is more specific than a source of substrate, and cannot be completely substituted for by the exogenous application of sucrose. The initiation of PS I as measured by this assay system, occurred between 6 and 9h after illumination and this could also be attributed to a delay in enzyme synthesis. In a recent paper Haslett and Cammack (1976) showed that ferredoxin-$NADP^+$ reductase activity in greening bean leaves increased exponentially after an initial lag between 5 and 15h and was dependent upon light intensity. These results confirm the theory that ferredoxin-$NADP^+$ reductase could be located on the outside of the thylakoid membrane (Berzborn, 1969) and is the last part of the electron transport system to be developed (Bradbeer et al., 1969; Gyldenholm and Whatley, 1968).

In vitro PS I mediated cyclic phosphorylation was detected in chloroplasts isolated from both photosynthetically competent and incompetent plants. The rates of activity were lower in the CMU/sucrose treatment than in the water control in spite of substantially higher chlorophyll contents and more advance chloroplast fine structure. Inhibition of cyclic phosphorylation in chloroplasts isolated from CMU/sucrose treated leaves may have been due to over-oxidisation of

an intermediate of the cyclic chain (Whatley, 1963) under the aerobic conditions in which the assay was conducted. Little change in the rates was observed, however, when the assay was carried out under nitrogen. Since anaerobic stimulation of cyclic phosphorylation could not be detected it is possible that electron flow is mediated through an endogenous component which requires the co-operation of PS II and hence complete electron flow, for its development.

Addition of CMU to photosynthetic green chloroplasts totally inhibits PS II electron flow when measured by reduction of conventional 'Hill' electron acceptors such as ferricyanide and DCIP (Wessels and Van Der Veen, 1956). Furthermore illumination of CMU treated etiolated pea explants in the presence of exogenously applied sucrose produced green plants in which the PS II as measured by ferricyanide reduction was inactive (figure 20). In the latter case it was difficult to establish whether PS II was inactive in these plants because CMU was occupying a site which was preventing electron flow, or whether the development of one or more of the PS II components was inhibited. A comparative analysis of the pigment compositions of both CMU/sucrose and control explants showed that the chlorophyll contents were similar and that the ratio of chlorophyll a:b remained unchanged. It is now generally accepted that the xanthophylls are an integral component of PS II (Ogawa et al., 1966; Wessels et al., 1973).Although lutein was present in substantial levels in etiolated leaves (figure 17) the onset of new synthesis was not observed until a point which correlated well with the onset of PS II activity. Changes in chloroplast ultra-structure were also similar in both treatments and onset of grana

formation was concomitant with PS II activity in the control. Although PS II is often present in plants without grana (see Discussion section 1) there is no evidence to suggest that grana stacking can occur in the absence of a functional or potentially functional PS II. The synthesis of chlorophyll-protein complex II, however, now appears to be no indication of PS II activity and can be induced in response to an active PS I (figure 28).

Manganese has a specific role in maintaining photosynthesis and its site of action has been placed on the oxidant side of PS II (Cheniae, 1970). More specifically manganese is thought to act closely to the oxygen evolving centres and may be the cofactor for the metallo water splitting enzyme (Cheniae, 1970). A secondary structural function has also been attributed to manganese (Homann, 1967) which is shown by the levels present (figure 22) in the etioplast prolamellar bodies. The CMU treated explants which were unable to develop functionally and which showed a marked decrease in membrane content after 24h illumination (plate 14) also showed a loss in manganese content. It is assumed therefore that the increase in manganese in the chloroplast membranes during greening in the other treatments was due to its incorporation into the oxygen evolving centres of PS II. Oxygen evolution as measured by the more recently discovered Hill electron acceptor, silicomolybdate (Giaquinta and Dilley, 1975) correlates well with the time course for manganese incorporation. The use of this assay demonstrated beyond all doubt that PS II in CMU/sucrose treated pea explants could be developed in

the absence of a functional electron transport system. The rates of PS II activity in the CMU/sucrose treatment on a chlorophyll basis were inhibited to a point below those of the water control. This may be explained by the fact that silicomolybdate has two sites at which it is able to accept electrons (Giaquinta and Dilley, 1975). CMU blocks PS II electron flow between these two sites and therefore activities were only half maximal. It would appear therefore that the oxygen evolving capacity of PS II can develop normally without total electron transport provided there is an alternative source of substrate present.

In conclusion, exogenously applied substrate can, in the majority of cases, substitute entirely for photosynthesis in chloroplast development. Therefore, the role of photosynthesis in chloroplast development is to supply carbohydrate as a potential source of energy for the synthesis of new membrane components and may be regarded as being non-specific. PS I activity as measured by $NADP^+$ reduction is impaired by the inhibition of photosynthesis during chloroplast development and it is suggested that complete electron flow and photosynthetic competence is necessary for the further synthesis of ferredoxin-$NADP^+$reductase. Furthermore <u>in vitro</u> PS I mediated cyclic phosphorylation is inhibited in chloroplasts isolated from CMU/sucrose treated explants. The rates could not be further stimulated under anaerobic conditions which would suggest that photosynthesis is specifically required for the synthesis of a PS I component. It is possible that the lack of this component is also responsible for the lag in the onset of PS I activity as measured by ascorbate photooxidation.

and exhibited by the CMU/sucrose treatment (see Discussion section 1). Furthermore if this component was present in CMU treated explants PS I induction might well be established.

3. THE ROLE OF CYCLIC PHOSPHORYLATION IN CHLOROPLAST DEVELOPMENT

Cyclic photophosphorylation can be demonstrated in chloroplasts isolated from higher plants only when cofactors of electron transport are added in catalytic amounts. Among these cofactors PMS promotes the highest rates of photophosphorylation (Jagendorf and Avron, 1958). Other redox substances such as FMN and the vitamins K_3 and K_5 (Whatley et al., 1955; Arnon et al., 1955) are also efficient cofactors of photophosphorylation although these require the presence of oxygen, and phosphorylation catalysed by them is inhibited by CMU (Krogmann and Vennesland, 1959). It has been suggested that these latter cofactors are mediating an oxygen dependent pseudocyclic phosphorylation.

There is considerable evidence for cyclic phosphorylation in vitro (see Introduction section 5.2) and in addition the results from this study have shown that it will operate in chloroplasts isolated from non-photosynthesising leaves. However, evidence for the presence of an in vivo system has been suggested largely on the basis of indirect evidence. Phillis and Mason as early as 1937 showed that light-induced starch synthesis from glucose could occur under anaerobic conditions. Under these conditions both oxidative phosphorylation and photosynthetic CO_2 uptake would have been inhibited

and an interpretation of these results would suggest an alternative source of ATP synthesis. These results were subsequently confirmed by Machlachlan and Porter (1959) who demonstrated that starch could be synthesised by tobacco leaf discs *in vaccuo*, to levels which were similar to those obtained under light aerobic conditions. Conflicting evidence was provided by Krall and Bass (1962). In their study anaerobic conditions were provided by a stream of highly purified nitrogen and starch synthesis from glucose was totally inhibited. Moreover, total inhibition of starch synthesis was exhibited by addition of CMU. Forti and Parisi (1963), however, demonstrated a substantial light-induced increase in the ATP content of saxifrage leaves treated with CMU and maintained under anaerobic conditions. Inhibition of both photosynthesis and oxidative phosphorylation by these means would suggest that ATP formation could only have been effected by cyclic photophosphorylation. Furthermore Antimycin A, an inhibitor of cytochrome b6 (Tagawa et al., 1963) and hence cyclic phosphorylation, was found to inhibit photoassimilation of glucose in *Chlorella* by 70% (Tanner et al., 1965). Similarly, glucose uptake was inhibited by Salicylaldoxime, itself thought to be a specific inhibitor of cyclic phosphorylation (Tanner et al., 1969). The synthesis of larger amounts of chloroplast protein (Dale, 1967) and more specifically isocitrate lyase (Syrett, 1966) has also been shown to occur under conditions when both photosynthesis and oxidative phosphorylation had been inhibited.

Further evidence for the presence of *in vivo* cyclic phosphorylation is suggested in this study. The light intensity

threshold for the conversion of substrate to chlorophyll in pea was shown in the CMU treatment to be at $1 W/m^2$ (figure 13). The exogenous addition of substrate in the form of sucrose to this treatment, not only substantially increased the total chlorophyll content after 48h illumination but also changed the light intensity at which maximum chlorophyll synthesis occurred. The light dependence of chlorophyll synthesis in this CMU/sucrose treatment cannot be due to photosynthesis, but could possibly be explained by the operation of in vivo cyclic phosphorylation (Dodge et al., 1971) and utilisation of resultant energy for chlorophyll synthesis. The maximum rate of chlorophyll formation was attained at the relatively low light intensity of $3 W/m^2$ and in this respect resembled the DCMU insensitive photoconversion of glucose to starch and cyclic phosphorylation dependent isocitrate lyase synthesis (Syrett, 1966). The low light saturation of cyclic phosphorylation has also been pointed out by Tanner et al. (1969).

These results strongly suggest that cyclic phosphorylation does exist in vivo in both higher plants and algae. The physiological role of the process, however, remains somewhat more elusive. According to the Calvin cycle 3 moles of ATP are necessary for each mole of CO_2 fixed in carbohydrate. For many years it was generally assumed that cyclic phosphorylation supplied 1 mole of ATP and non-cyclic phosphorylation supplied two moles (Arnon et al., 1967). Tanner et al. (1969), showed that cyclic phosphorylation in spinach leaves could be inhibited by salicylaldoxime without a reduction in oxygen evolution or photosynthetic CO_2 uptake.

The possible role of cyclic phosphorylation in early

chloroplast development has been suggested in the previous sections of the Discussion. It has been shown that the initial photoconversion of protochlorophyll(ide) to chlorophyll, the dispersal of the prolamellar body and the lag phase, are non-energy requiring processes. During the lag phase PS I components which are present in the etioplast, and therefore require no synthesis, are inserted into the membrane. It is possible that at this stage PS I mediated cyclic phosphorylation is able to function, utilising the small levels of chlorophyll which are present after the initial photoreduction of protochlorophyll(ide). Thus energy which is conserved by cyclic phosphorylation could be used to synthesise small levels of chlorophyll-protein complex II and additional chlorophyll (figures 28 and 14). As a result, higher levels of energy are utilised in the further development of PS I, the synthesis of PS II components, and hence new membrane. The onset of PS II could result in the operation of non-cyclic phosphorylation which could be utilised for additional chlorophyll synthesis, since the enzyme system for the formation of reducing power may be limiting (Discussion section 1). It is suggested, therefore, that by utilising the process of cyclic phosphorylation chloroplast development can be autonomous.

4. EXCITATION ENERGY SPILLOVER

Present evidence on the interaction of the two photosystems would suggest that there is an exchange of excitation energy (spillover). In the 'spillover' model, light energy which is absorbed but not used by PS II, is transferred to PS I. Since the light absorbed by PS I is of a lower energy the reverse situation cannot occur. A comparison between the rates of photochemical activities in chloroplasts isolated from illuminated intact and excised plants suggests the occurrence of spillover of excitation energy between PS II and PS I (Table 1). Explants, although containing adequate substrate, show an approximate 30% decrease in chlorophyll content, PS II activity, and whole plant CO_2 uptake. These results would suggest that although the excised plants are supplied with an adequate energy supply, normal chloroplast development is inhibited by the lack of a growth factor which is translocated to intact plants from the cotyledons. Nevertheless, PS I activities as measured by ascorbate photooxidation showed the converse, and the rates of PS I activity in the explants were approximately 40% above those of the intact plants. It is suggested that if PS II is impaired during development the excess excitation energy is transferred and utilised by PS I. A further example of spillover was shown in the PS II inhibited CMU/sucrose treatment (figure 23). After 30h illumination the rates of PS I activity continued to increase over and above those of the water/sucrose treatment. Moreover, Pallett(unpublished) has demonstrated that addition of CMU to greened flax cotyledons produced increases in PS I activity which were proportional to the amount of CMU inhibition of

PS II.

In whole organisms the control of the distribution of electronic excitation can be effected by illumination with light absorbed preferentially by one or by the other photosystem (Papageorgiou, 1975). Several minutes of illumination with PS II light was shown to be sufficient to transform a photosynthetic organism into a state (light II State) in which fewer quanta were delivered to the PS II, than at the beginning of the illumination period. Excess light quanta, when the organism is in light II State, were transferred to PS I. Prolonged darkness, however, or prolonged illumination with PS I light, led to a state (light I State) in which a larger proportion of the available excitation energy was transferred to PS II.

Active thylakoids are presently accepted to be dynamic entities in which the structural and functional characteristics respond to environmental stimuli. Illumination of chloroplasts induces a large ionic movement across the thylakoid membranes which affects both the configuration of the thylakoids themselves, and the conformation of the thylakoid membranes. Moreover, in response to the electric field set up across illuminated lamellae (Junge and Witt, 1968), protons are transferred from the surrounding stroma to the thylakoid interior, while metal cations move outside to maintain the electrical and osmotic equilibrium (Jagendorf and Hind, 1963; Dilley and Vernon, 1965; Nobel, 1967). Murata (1970) correlated these changes in the metal cation concentration in thylakoids with the light I and light II State interconversions, which control the distribution of chlorophyll a excitation between the two photosystems. It was

suggested that at light II State the thylakoids were depleted of metal cations and thus the spillover of excitation energy to PS I proceeds uninhibited. When light I State was induced, the thylakoid was rich in metal cations, spillover from PS II to PS I was blocked and consequently the efficiency of PS II was increased.

The spillover from PS II to PS I demonstrated by excised plants can be explained according to this theory. The normal development of PS II during greening requires one or more factors which in intact plants are translocated from the cotyledons. PS I as measured by ascorbate photooxidation is unaffected by excision and will develop normally in the presence of sucrose. As already discussed in the previous section, components of PS I, which might be affected are between ferredoxin and $NADP^+$; but these were bypassed in this assay. Illumination of the chloroplasts isolated from explants resulted in the overloading of the PS II electron traps and the chloroplasts were transformed to a light II State. It is further suggested that excess excitation energy was transferred to PS I and hence the rates of PS I activity were increased above those of the intact plants which have normally developed PS II. Similar reasoning can be applied to explain the high rates of PS I activity demonstrated by CMU/sucrose treated pea explants. Inhibition of PS II electron flow by CMU, produced a shortage of free electron traps. This condition was similar to that caused by saturating the electron trap of PS II by PS II light, and hence produced a light II State. Spillover of excitation energy from PS II to PS I, therefore, proceeded uninhibited.

The transformations from one state to another can be regarded

as a safety mechanism and allows a plant to adapt to changeable physiological conditions without major adaptive changes in chemical composition of its light harvesting apparatus. Moreover, it is possible that a loss in non-cyclic energy production due to depression of PS II activity, might be compensated for by an increase in cyclic phosphorylation mediated by PS I. In this present study, however, it might be expected that the non-functional chloroplasts adjusted further to cope with the increased dependence upon PS I. It has been shown that the pigment composition of plants is adjusted to meet the physiological strains imposed by the quality and/or quantity of illumination (Ghosh and Govindjee, 1966). A corresponding increase in β-carotene, associated with the PS I of CMU/sucrose greened plants, would seem appropriate to absorb much of the potentially destructive power of light. Figure 16 showed that no further increases in β-carotene occurred, and it must be concluded that the carotenoids present are sufficient to protect the chlorophylls (Krinsky, 1971).

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Electron Transport Capabilities of Chloroplasts Isolated from Non-photosynthesizing Leaves*

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Summary. The photosynthetic inhibitor CMU was used to retard chlorophyll formation in illuminated dark grown pea (*Pisum sativum* L. var. Meteor) explants. Levels of chlorophyll were restored to those of the control plants by the addition of exogenous sucrose, but carbon dioxide uptake was absent. *In vitro* electron transport studies with chloroplasts isolated from these plants revealed a functional PS I and PMS catalysed cyclic photophosphorylation. PS II activity as measured by ferricyanide reduction was absent but was fully functional in the presence of silicomolybdate. The results showed that a functional PS II developed in the absence of a complete electron transport system and that cyclic phosphorylation could operate *in vivo* without PS II.

Introduction

Numerous investigators have followed the development of CO_2 uptake or the partial reactions of electron transport of isolated chloroplasts during the illumination of etiolated leaves. The commencement and further development of photosynthetic activity appears to follow the progressive development of chlorophyll. When chlorophyll formation was retarded by the addition of the photosynthetic inhibitor CMU, the effect could be reversed by the addition of exogenous sucrose (Klein and Neumann, 1966; Dodge *et al.*, 1971). In this study we have used this experimental system to compare the development of the functional capabilities of chloroplasts isolated from photosynthetically active and photosynthetically inhibited leaves.

* *Abbreviations:* CMU = 3-(4-chlorophenyl)-1,1-dimethylurea; DCMU = 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PS I = Photosystem I; PS II, Photosystem II; PMS = Phenazine methosulphate.

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Materials and Methods

Pea seeds (*Pisum sativum var.* Meteor) were grown on Levington compost for 6 days at 25°C in continuous darkness. Etiolated pea shoots 5 cm long were detached and placed in vials containing sucrose and CMU as required. Experimental manipulations were carried out under a green safe light. A 16 h dark period preceeded illumination of 6 W/m^2 for 24 h.

The total chlorophyll content of leaves and chloroplast preparations was obtained by the method of Arnon (1949).

CO_2 exchange of plant material was measured by using an infra-red gas analyser (Grubb Parsons, Ltd.) as described by Dowdell and Dodge (1970).

Chloroplasts were prepared using the methods and media of Izawa and Good (1968). Photoreactions were carried out in an oxygen electrode (Rank Bros.) at 20°C. Illumination was provided by a 1,000 W lamp giving 280 W/m^2 at the reaction chamber. The activity of PS II was estimated by following oxygen evolution during silicomolybdate (Barr *et al.*, 1975) or ferricyanide reduction. Standard reaction mixtures of 3 ml contained 90 µmol tris buffer pH 8.0, chloroplasts containing 150 µg chlorophyll where possible and 1 mg silicomolybdate or 2 µmol ferricyanide as required. PS I activity was estimated by ascorbate photoxidation. This was followed as an oxygen uptake with a reaction mixture of 3 ml containing 90 µmol tris buffer pH 8.0, 40 µmol sodium iso-ascorbate, 0.2 µmol DCIP, 0.02 µmol methyl viologen and chloroplasts containing 80 µg chlorophyll. Cyclic photophosphorylation was estimated by the method of Dowdell and Dodge (1970) incorporating the inorganic phosphate assay of Ames (1966).

Results and Discussion

Table 1 shows that chlorophyll formation which was almost totally inhibited by the presence of CMU could be restored to a value greater than in the untreated leaves by the addition of sucrose. An apparently normal chlorophyll formation was induced under these circumstances although the explants were totally incapable of carbon dioxide uptake.

Table 2 shows some of the electron transport capabilities of chloroplasts isolated from normal and CMU/sucrose treated non-photosynthesizing leaves. PS II activity was measured as an oxygen evolution

Table 1. Chlorophyll formation and carbon dioxide uptake in greening leaves after 24 h illumination

	Treatment of leaves		
	H_2O	CMU 5×10^{-4} M	CMU 5×10^{-4} M sucrose 5×10^{-2} M
Chlorophyll mg/g	0.194	0.024	0.269
CO_2 uptake µl/g fresh wt./h	151.33	0.00	0.00

Table 2. Reactions of chloroplasts isolated from greening leaves after 24 h illumination

Reaction	Treatment of leaves	
	H_2O	CMU 5×10^{-4} M sucrose 5×10^{-2} M
O_2 evolved (ferricyanide) µmol O_2/g fresh wt./h	13.5	0
O_2 evolved (silicomolybdate) µmol O_2/g fresh wt./h	11.5	8.2
Ascorbate photooxidation, µmol O_2/g fresh wt./h	48.47	42.19
Cyclic photophosphorylation µmol Pi/g fresh wt./h	330.7	328.74

with ferricyanide or silicomolybdate as electron acceptors. With ferricyanide as oxidant, chloroplasts from CMU/sucrose treated leaves were totally inhibited. In contrast, with silicomolybdate as oxidant, the rate of oxygen evolution was considerable, although not quite that of the control leaves. Barr *et al.* (1975) have recently demonstrated that silicomolybdate reduction in normal chloroplasts is unaffected by the related urea herbicide DCMU. This oxidant appears to be reduced at a point prior to the site of action of this and related inhibitors (Dodge, 1975). PS I measured as ascorbate photoxidation or cyclic photophosphorylation in the presence of PMS, developed almost normally in the CMU/sucrose treated leaves (Table 2). Little information is currently available into the functional integration of chloroplast electron transport. This present investigation demonstrates that the organisation of PS II and the oxygen evolving system proceeds independently of a functional electron transport system. It also shows that PS I could operate *in vivo* in the absence of PS II and that cyclic photophosphorylation might play a part in chloroplast development (Dodge *et al.*, 1971).

Further work is in progress to follow the developmental sequence of electron transport capabilities and also the structural organisation of the different chloroplasts.

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